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An investigation in to the cellular basis of tendon degeneration.

AN INVESTIGATION INTO THE CELLULAR BASIS OF TENDON DEGENERATION

Helen Lucy Birch

A thesis submitted to the University of Bristol in accordance with the requirements for the degree of Ph.D. in the Faculty of Medicine, Department of Anatomy.

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Abstract

1. Extracellular matrix composition and cellularity were analysed in equine superficial digital flexor tendons (SDFT) collected from Thoroughbred horses and compared with a flexor tendon which rarely shows degenerative change and subsequent injury (deep digital flexor tendon, DDFT). Collagen content did not differ between the two flexor tendons however, there were substantial differences in collagen type and organisation. The SDFT had a higher type III collagen content, higher levels of the mature trifunctional collagen crosslink hydroxylysylpyridinoline, lower total chondroitin sulphate equivalent glycosaminoglycan content, smaller diameter collagen fibrils and a higher cellularity than the DDFT. Ageing in both tendons resulted in an increase in collagen-linked fluorescence and a decrease in cellularity in the DDFT but not the SDFT. No differences were apparent in this study between SDFT from exercised and un-exercised horses.

2. A comparison of extracellular matrix composition and cellularity was made between a group of normal tendons and a group of degenerated tendons. Degenerated tendons were those showing a macroscopic alteration to the central core characterised by a reddish stain visible in transverse sections but no change in tendon shape or size. Degenerated tendons showed an increase in total chondroitin sulphate equivalent glycosaminoglycan content, high levels of type III collagen, a decrease in collagen linked fluorescence and high cellularity suggesting an increased matrix turnover in these tendons.

3. Oxidative energy metabolism was investigated in freshly isolated and in cultured equine superficial digital flexor tendons. Freshly isolated cells possessed activities of the mitochondrial enzymes citrate synthetase (CtS) and glutamate dehydrogenase (GDH) at levels similar to those for other mammalian cells with well defined oxidative metabolism. Similar levels of CtS and GDH were also apparent in primary cultured cells obtained from explants of equine tendons. Glucose utilisation and oxidation were demonstrated in both freshly isolated and explant-derived cells. Cultured cells were shown to maintain levels of ATP similar to those maintained by a wide range of mammalian cells, and these levels were diminished by incubation with a mitochondrial respiratory un-coupler, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (5 μM, FCCP). Taken together these data demonstrate that tendon cells are capable of oxidative energy metabolism and that this is largely maintained in cultured cells. Oxygen-dependent substrate metabolism is thus likely to be important in maintaining normal cell function and could represent a pathway for tissue damage.

4. The effects of hypoxia and free radicals on cell growth rates and collagen production was studied *in vitro* using equine tendon fibroblasts cultured from explants of SDFT tissue. Hypoxia was simulated by the addition of a respiratory un-coupler to the medium and free radicals generated by the addition of H_2O_2 or glyceraldehyde to the medium. Cell growth rates were not significantly reduced below a concentration of 10 μM FCCP and 10^{-5} M H_2O_2 or 10^{-4} M glyceraldehyde. Free radicals (at a concentration just less than that which causes cell death) had no effect on collagen secretion. FCCP (5 μM) resulted in a decrease in type III collagen secretion into the medium while type I collagen secretion was unchanged.

5. The effect of hyperthermia on tendon fibroblast function was studied i*n vitro* using cells cultured from explants of equine SDFT. Tendon fibroblasts were significantly more resistant to hyperthermia at 45°C than skin fibroblasts (grown by the same method from explants of skin overlying the SDFT) and NRK49F cells. Tendon fibroblast survival fraction was not significantly decreased until 60 min. of heating at 45°C. A 10 min. heat shock at 45°C resulted in an increase in cell proliferation rate and an increase in both type I and III collagen secretion into the medium.

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Memorandum

This thesis represents the unaided work of the author, except where acknowledged overleaf. The views expressed in the dissertation are those of the author and not necessarily those of the University.

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INTRODUCTION

1.1 General Introduction

The Thoroughbred horse is an impressive athlete. For example, a fit horse is able to maintain a speed of about 41 mph over a distance of half a mile whereas a human athlete can only average 17 mph over the same distance. This is because the horse has evolved with an optimisation of structural and physiological features which enable it to achieve peak athletic performance.

Many racehorses and other competition horses however, are lost from training each year due to partial rupture of the flexor tendons (Webbon, 1973), particularly the superficial digital flexor tendon (Webbon, 1977). Once rupture of this tendon has occurred the victim rarely makes a successful return to competition and these horses are usually destroyed. The equine superficial digital flexor tendon has a remarkably high tensile strength and rupture occurs only following a degenerative change which results in a weakening of the tendon (Forsell, 1952). A similar injury, where a degenerative change precedes rupture (Kannus & Józsa, 1991), is commonly seen in the Achilles tendon of human athletes involved in competitive sports (Smart *et al.*, 1980) as well as those in recreational sport and day-to-day activities (Hess *et al.*, 1989).

Muscle and bone are able to adapt to their mechanical environment and undergo hypertrophy in response to training, thereby increasing in mass (Wolff's Law). Whether such changes occur in tendon, which forms the link between muscle and bone, however, is largely unknown. Thus, the response of tendons to training and the effect of ageing is of intrinsic interest. In addition, the considerable public interest in racing and the welfare of the racehorse, as well as the financial loss incurred by wastage due to equine flexor tendon injury, further emphasise the need for greater understanding and hence prevention of this problem. Training programmes must be designed to reduce the risk of injury to the equine, as well as human, athlete and this requires an understanding of the adaptive response of tendon tissue at the molecular and cellular level.

This thesis postulates that the aetiology of tendon degeneration is mediated by cellular damage. Chapter one will present a review of the current literature relating to tendon structure and function with particular emphasis on equine flexor tendons and human Achilles tendon. A number of areas are highlighted and a series of experimental hypothesis developed which will be tested in the subsequent chapters.

Fig. 1.1 Photograph of a galloping horse showing mid-stance extension of the metacarpo-phalangeal joint.

1.2 Evolution

The evolutionary adaptations of the horse have been described by Hildebrand (1974). The horse, a prey animal, is able to run at high speeds and the anatomy of its limbs have evolved to do so with maximum efficiency. This has been achieved by elongation of the leg, and particularly the most distal segments. The result is that the horse stands on what is equivalent to the human fingernail; i.e. it's hoof. In addition the limbs have been made as light as possible; there are no muscles below the carpus in the horse. Bulky muscles have been displaced from their "site of action" in the lower limb and are instead connected to their joints by long flexor tendons.

1.3 Anatomy

Three main collagenous structures support the metacarpo-phalangeal joint in the horse (fig. 1.2). These are the interosseous muscle, the deep digital flexor tendon and the superficial digital flexor tendon. The interosseous muscle, or suspensory ligament, is attached at its proximal end to the caudal surface of the third metacarpal bone close to the carpus. It passes distally on the palmer aspect of the third metacarpal and divides into two branches each of which inserts on to one of the paired proximal sesamoid bones. The suspensory ligament also has extensions which pass either side of the proximal phalanx and join the common digital extensor tendon. The deep digital flexor tendon (DDFT) and superficial digital flexor tendon (SDFT) originate from the bellies of the deep digital flexor muscle and superficial digital flexor muscle respectively which are located on the caudal aspect of the radius. The SDFT passes through the carpal canal, where it is enveloped by the carpal tendon sheath. The distal reflection of the carpal synovial sheath is about 6 cm below the carpal joint. The two tendons pass distally on the caudal aspect of the metacarpo-phalangeal joint running in the groove provided by the sesamoid bones and intersesmodian ligament. The DDFT passes over the distal sesamoid bone and spreads out in a fan-like fashion inserting on the underside of the distal phalanx. The SDFT widens out at the distal end of the third metacarpal bone to form a ring completely encircling the DDFT. Below the metacarpo-phalangeal joint the SDFT divides into two parts attaching to the proximal and middle phalanx. The SDF and DDF tendons both receive accessory ligaments, the superficial from above the carpus to the caudal surface of the radius and the deep from below the carpus to the caudal surface of the third metacarpal bone.

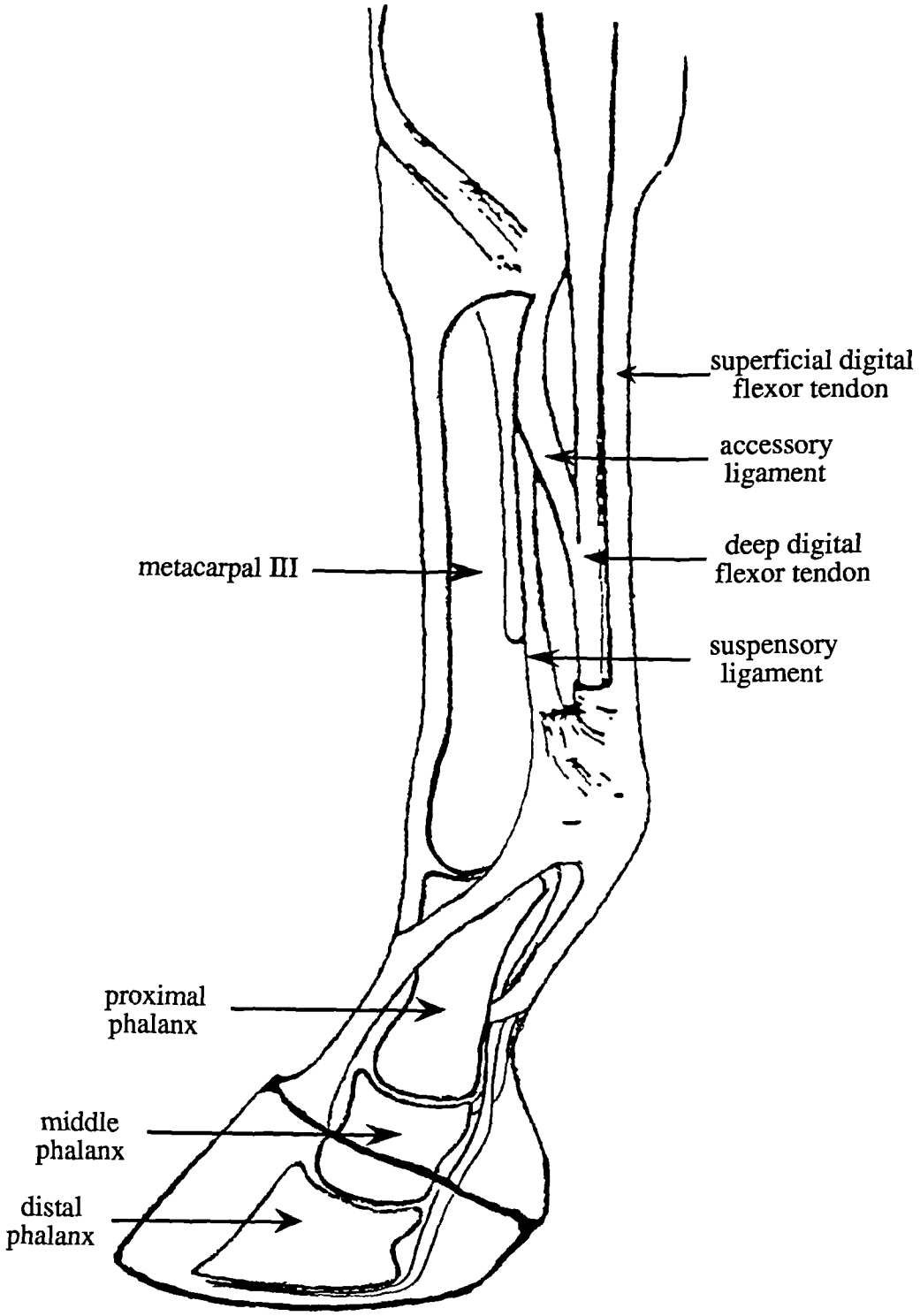


Fig. 1.2 Anatomy of the distal part of the equine forelimb.

1.4 Structure of Tendon

Tendon is one of many functionally and structurally diverse connective tissues. All connective tissues are composed of four main types of macromolecules; these being collagen, elastin, proteoglycans and connective tissue glycoproteins. Collagen and elastin form the fibrous scaffolding of the connective tissue, whilst the proteoglycans and glycoproteins form the ground substance filling the interstices and interfaces between the cells and the fibrous element (Labat-Robert *et al.*, 1990). The macromolecules of the ground substance are now known to have very specific cell-matrix and cell-cell interactions (Labat-Robert *et al.*, 1990).

The tendon is composed of bundles of collagen fibres lying in parallel with the longitudinal axis of the tendon, with little if any, elastin present. Rows of tendon fibroblast cells lie alongside the collagen fibres embedded in the ground substance of the tendon (Birk & Trelstad, 1986). The whole of the tendon is surrounded by a thin connective tissue layer; the epitenon. This is continued into the tendon as the endotenon where it separates tendon bundles and carries blood vessels, lymphatics and nerves. Where there is no synovial sheath the tendon is surrounded by a layer of loose connective tissue termed the paratenon (Elliott, 1965).

1.5.1 Collagen

Collagen is the main constituent of tendon forming about 80% of the dry weight in bovine Achilles tendon (Koob & Vogel, 1987a). At present fourteen different types of collagen have been identified (van der Rest & Garrone, 1991). Tendon however, is comprised predominantly of type I collagen with a small amount of type III also present and therefore the following review will refer to type I and III collagens.

The collagen molecule is the basic unit for the collagen fibre. The existence of such a unit was first postulated by Gross (1956) and named tropocollagen. The tropocollagen or collagen molecule is composed of three polypeptide chains wound into a triple helix (fig 1.3). Each chain is a left-handed helix with three amino acid residues per turn. The chains are then wound around each other in a right handed superhelix with a pitch of about 39 residues (van der Rest & Garrone, 1991). In order to form this triple helix, collagen has a unique primary structure.

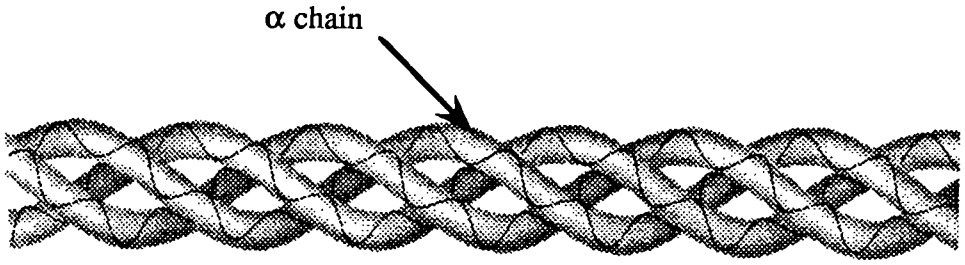


Fig. 1.3 Portion of a right-handed superhelix tropocollagen molecule. (Darnell *et al.*, 1986)

Primary structure

The triple helix is composed of a repeating amino acid triplet; Gly-X-Y (Grassmann *et al.*, 1965). The amino acid glycine therefore, forms about a third of the amino acid residues. The reason for this high glycine content is the absolute requirement of glycine every third residue for the triple helix structure; the centre of the helix cannot accommodate an amino acid with a larger side chain than the H of glycine (Ramachandran & Kartha, 1954). The X and Y positions are often occupied by the imino acid proline and its hydroxylated analogue 4-hydroxyproline, the hydroxylated form of proline usually occurring in the Y position (Segal, 1969). Lysine residues also frequently occur and are sometimes hydroxylated to give 4-hydroxylysine, some of the hydroxylysine residues are then glycosylated. There are very few aromatic amino acids along the length of the polypeptide chain. Side chains of the X and Y residues are directed outwards where they participate in both intra- and intermolecular interactions. In both type I and III collagen each polypeptide chain is composed of approximately 330 amino acid triplets. At the ends of the triple helical domain are short non-helical regions, 15 AA at the N-terminal end and 26 AA at the C-terminal. These non-helical regions are termed telopeptides and their composition varies slightly between collagen types. Type I collagen is made up of two identical chains $\alpha 1$(I) and one $\alpha 2$(I) chain (Piez *et al.*, 1963). Type III collagen is made up of three $\alpha 1$(III) chains (Miller *et al.*, 1971).

Stabilisation of the collagen triple helix

The triple helix is stabilised by intramolecular hydrogen bonds, one per triplet from the NH of the glycine to the back-bone C=O of the residue in the X position in the adjacent chain. Thus each molecule is stabilised by about 1000 such hydrogen bonds. In addition the lack of rotation around the Cα-atom of proline results in a fairly stable triple helix conformation (fig. 1.4).

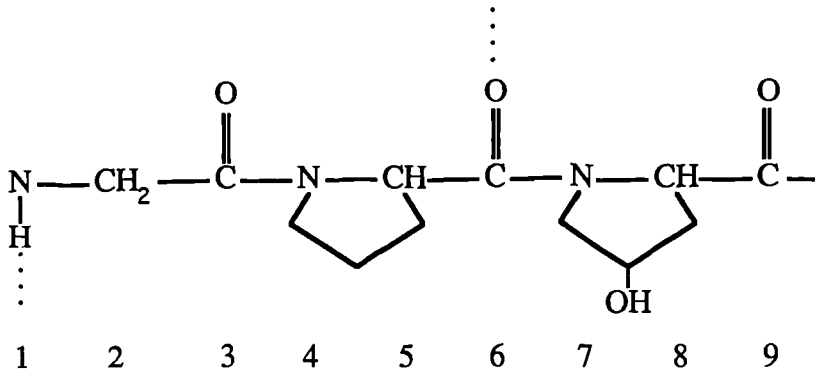


Fig. 1.4 The triplet sequence Gly-Pro-Hyp illustrating elements of collagen triple helix stabilisation. The numbers identify peptide backbone atoms. The conformation is determined by *trans* peptide bonds (3-4, 6-7 and 9-1); fixed rotation of bond in proline ring (4-5); limited rotation of proline past the C=O group (bond 5-6); interchain hydrogen bonds (*dots*) involving the NH hydrogen at position 1 and the C=O at position 6 in adjacent chains; and the hydroxy group of hydroxyproline, possibly through water-bridged hydrogen bonds.

The "melting temperature" of collagen is the temperature at which the triple helix unwinds and is generally 2 - 3°C above the normal physiological temperature. Variation in melting temperature is due to variation in the content of proline and hydroxyproline. The most stable triplet is Gly-Pro-Hyp (Segal, 1969). A triple helix formed by non-hydroxylated α chains exhibits a transition temperature of about 15°C lower than the melting temperature of a triple helix of normal, hydroxylated collagen (Berg & Prockop, 1973). Hydroxyproline, therefore also plays an important role in the stabilisation of the triple helix. The reason for this increased stability following hydroxylation of proline is not really known. One suggestion is that the hydroxy group is involved in a water bridged hydrogen bond between chains (Pineri *et al.*, 1978).

1.5.2 Collagen Synthesis

The genes coding for the fibrillar collagens are remarkably similar in intron/exon structure showing a high degree of conservation with evolution (Boedtker *et al.*, 1983). In general the genes are large for the fibrillar collagens (18-39 kb), with only some 10-30% of the gene coding for the protein (Chu *et al.*, 1984; Boedtker *et al.*, 1983). Like most genes, those for collagen contain a 5' promoter region which contains the usual TATA and CCAAT boxes important in RNA polymerase binding. In addition,

other functional regions have been identified which bind DNA binding proteins that regulate gene expression (Vuorio & de Crombrugghe, 1990).

Collagen is synthesised initially as a pre-procollagen with quite large N- and C-terminal globular domains (fig. 1.5). Like all proteins secreted from the cell the polypeptide chain contains a short hydrophobic signal peptide (23 AA) at the N-terminal end. This allows the nascent α-chains to penetrate the rough endoplasmic reticulum (Kühn, 1987).

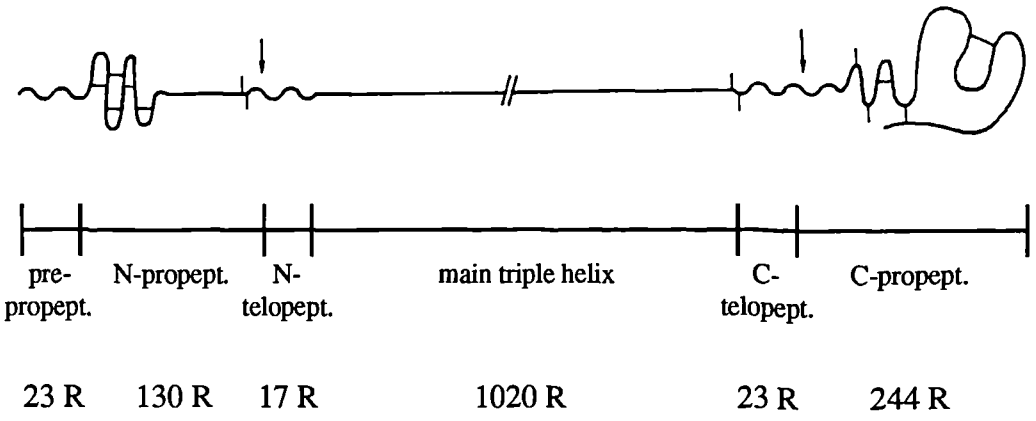


Fig. 1.5 Schematic representation of the prepro α1 (III) chain of collagen (Kühn, 1987). The arrows indicate the cleavage sites of the amino and carboxyl procollagen peptidase. R, amino acid residues.

The signal peptide is removed by the signal peptidase enzyme. This initial gene product undergoes extensive post-translational modifications which take place in the rough endoplasmic reticulum, Golgi organelles and the extracellular space after secretion (table 1.1). A crucial step in the biosynthesis of collagen is the hydroxylation of proline residues by prolyl 4-hydroxylase to form 4-hydroxyproline (Kivirikko & Myllylä, 1980 & 1985) and, to much lesser extent, by prolyl 3-hydroxylase to form 3-hydroxyproline (Cardinale & Udenfriend, 1974). Prolyl 4-hydroxylase requires oxoglutarate, O_2, ascorbate and ferric ions as cofactors. Hydroxylation of lysine residues to form hydroxylysine also occurs in the rough endoplasmic reticulum and is catalysed by the enzyme lysyl hydroxylase (Majamaa *et al.*, 1985). The hydroxylysine residues can then become glycosylated by the addition of galactose, a process catalysed by hydroxylysine galactosyl transferase, or by the addition of galactose then glucose the latter catalysed by galactosyl hydroxylysine glucosyl transferase (Spiro & Spiro, 1971a&b; Kivirikko & Myllylä, 1979). In types I and III collagen the level of

Table 1.1 Enzymatic steps and sequence of events in procollagen biosynthesis. (Davidson & Berg, 1980)

Location	Major events	Enzyme	Function
Rough ER	Signal sequence cleaved	Signal peptidase	Removal of hydrophobic signal peptide
	Hydroxylation of prolyl residues to 3-hydroxyprolyl	Prolyl 3-hydroxylase	?
	Hydroxylation of prolyl residues to 4-hydroxyprolyl	Prolyl 4-hydroxylase	Stability of triple helix
	Hydroxylation of lysyl residues to hydroxylysyl	Lysyl hydroxylase	Site for O-glycosylation
	Addition of N-linked high-mannose oligosaccharide	Lipid-linked oligosaccharide transferase	Secretion of protein
Golgi complex	Formation of triple helix	Self-assembly	Forms rigid collagen structure
	Addition of galactose to hydroxylysyl residues	Galactosyl hydroxylysyl transferase	?
	Addition of glucose to galactose	Glucosyl transferase	?
	Formation of inter- and intrachain disulphide bonds	?Disulphide isomerase	Stabilisation of procollagen structure
Extracellular	Cleavage of N-terminal pro sequence	N-terminal procollagen peptidase	Permits alignment of tropocollagen into fibrils
	Cleavage of C-terminal pro sequence	C-terminal procollagen peptidase	Permits alignment of tropocollagen into fibrils
	Oxidation of lysyl residues to aldehyde derivates	Lysyl oxidase	Site of formation of cross-links
	Formation of interchain cross-links	?Spontaneous	Stabilisation of fibres

glycosylation is low being only about 0·5 - 1% of the hydroxylysine residues. The function of the carbohydrate residues is not clear. They do however, affect the amount of associated water and hence the fibril spacing. All five post-translational enzymes can only modify the nascent collagen polypeptide chain, that is before formation of the triple helix occurs. The C-terminal propeptide contains eight cysteine residues, some of which are involved in interchain disulphide bonds catalysed by the enzyme protein disulphide isomerase. This aligns the three polypeptide chains in register and initiates triple helix formation (Fessler *et al.*, 1981; Uitto & Prockop, 1973).

The secretion of the newly formed procollagen molecule is an energy dependent process and is inhibited by colchicine suggesting the involvement of microtubules (Dehm & Prockop, 1972). The first step following exocytosis of procollagen is the cleavage of the N- and C-propeptides by N-terminal procollagen peptidase and C-terminal procollagen peptidase allowing the collagen molecules to aggregate into fibrils. The presence of the N- and C-propeptides prevent fibril formation within the cell. It has also been suggested that amino and carboxy propeptides may play an important role in the control of fibril formation and growth outside the cell. Type I and III molecules containing amino propeptide have been observed on the surface of thin collagen fibrils. Type I collagen fibrils with an increasing diameter lack the propeptide. Type III collagen fibrils which do not grow in diameter keep the amino propeptide on their surface (Fleischmajer *et al.*, 1981). There is also some evidence that the cleaved N-propeptide also acts as a feed back inhibitor of collagen synthesis (Wiestner *et al.*, 1979).

1.5.3 Packing of Collagen Molecules

The correct packing of collagen molecules is very important to give the connective tissue its mechanical strength. The added stability of the molecule due to molecular packing and association with other matrix molecules is demonstrated by the shrinkage temperature which, for fibrous collagens, is 27°C above the melting temperature of the collagen molecule (Kühn, 1987). The shrinkage temperature is defined as the temperature at which the collagen fibre shrinks to one quarter of its original length, if unrestrained.

Once the propeptides have been cleaved, the collagen molecules are able to self assemble into fibrils. Under the electron microscope collagen fibrils appear as banded structures with a repeating periodic pattern every 67 nm. This periodicity is referred to as D. The molecular length of 300 nm is represented by 4·4 D, where 0·4 D is the end-overlap distance (fig. 1.6). This organisation has been confirmed by electron microscope (Petruska & Hodge, 1964) and amino acid sequence studies (Trus & Piez, 1976; Hofmann *et al.*, 1978). It was found in a computer study that the 'best fit' for

hydrophobic and ionic interactions was at a position where the two chain sequences overlapped by 234 residues which is equivalent to the 67 nm observed in the electron micrographs. The longitudinal packing of collagen molecules is therefore directed by hydrophobic and hydrophilic interactions.

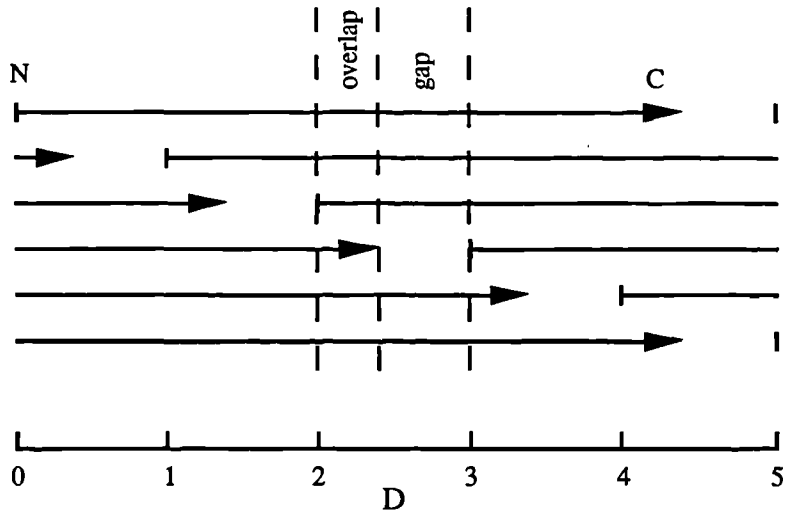


Fig. 1.6 A schematic representation of the parallel D staggered arrangement of collagen molecules within the fibril.

There is evidence for a well defined microfibrillar substructure in native collagen fibrils (Gelman *et al.*, 1979; Parry and Craig, 1979). Several types of structural model have been proposed with two stranded (Woodhead-Galloway *et al.*, 1975), four stranded (Veis & Yuan, 1975) five stranded (Smith, 1968; Miller & Parry, 1973) and eight stranded microfibrils (Nemetschek & Hosemann, 1973) on the basis of electron micrograph data and information provided by the medium angle X-ray diffraction patterns of fibrils. Later studies led to the suggestion of a new type of model based on quasi-hexagonal packing of collagen molecules (Hulmes & Miller, 1979; Trus & Piez, 1980; Fraser *et al.*, 1983). However, there is no well-defined microfibrillar substructure in this model and up to now results from electron microscope studies of collagen fibrils (Na *et al.*, 1986) have been explained by models with five stranded microfibrils.

Collagen fibrils, extending over hundreds of microns, exhibit remarkable uniformity of diameter and grow with pointed ends (Holmes *et al.*, 1991). The mechanisms directing the lateral packing of collagen molecules, unlike those involved in the longitudinal packing, are ill-understood. Holmes *et al.* (1991) in their "tapering end" model suggest that molecules add to the growing tips of collagen fibrils in a highly

regulated manner to generate a symmetrical growing tip extending several microns and such that the relative axial to lateral growth is controlled.

1.5.4 Collagen Crosslinking

The high mechanical strength of collagen fibres is brought about through the formation of intra- and intermolecular crosslinks. The importance of collagen crosslinks is illustrated by consideration of diseases such as lathyrism, dermatosparaxis in sheep and cattle or Ehlers-Danlos syndrome in man, where collagen cross-linking is inhibited. In these diseases lesions connective tissue lesions occur because the collagen matrix has little mechanical strength. There are three types of crosslink; disulphide bridges, lysyl oxidase mediated crosslinks and those formed by non-enzymatic glycosylation of lysine and hydroxylysine residues (Bailey & Light, 1989).

Disulphide bridges

Disulphide bridges are confined to collagen molecules with cysteine present, and of the common forms, only type III and IV do so. The cysteine residue responsible for crosslinking in the type III molecule is located in the final triplet of the triple helix before the non-helical C-terminal domain. Three cysteine residues are therefore present in each type III molecule. Thus, two of these may react to form an intramolecular disulphide bridge which may be catalysed at the stage of helix formation in the endoplasmic reticulum by the enzyme protein disulphide isomerase. The third cysteine residue would be free to form disulphide bridges with cysteine residues of neighbouring molecules although it has not been proven that this is the case. Disulphide bridges are not considered to be important in the mechanical functioning of collagen fibres.

Lysyl oxidase mediated reducible crosslinks

The second type of crosslink are the lysine derived reducible crosslinks. The first step in the biosynthesis of these crosslinks is the oxidative deamination of certain lysine and hydroxylysine residues by the enzyme lysyl oxidase, a copper metallo-enzyme requiring molecular oxygen. The extracellular enzyme, lysyl oxidase, binds to newly formed fibres and oxidatively deaminates the lysine residues in the non-helical N- and C-terminals of each α chain converting them to aldehyde groups. The $\alpha 2$ (I) chain does not have a lysine residue at the C-terminal domain. Thus, in type I collagen two lysine aldehydes are formed at the C-terminal and three at the N-terminal.

Two lysine aldehydes are able to react with each other to form an aldol condensation product between two α chains within one molecule, otherwise known as β_{12}. An intramolecular aldol exists at the N-terminus of type I collagen (between α1 and α2) leaving one lysine aldehyde at this end and two lysine aldehydes at the C-terminal free to form intermolecular crosslinks (Bailey & Light, 1989).

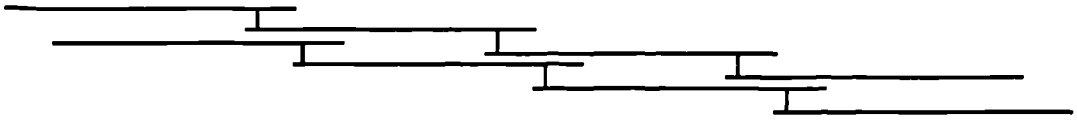


Fig. 1.7 Location of lysyl oxidase mediated crosslinks.

Intermolecular crosslinks are formed by the reaction of lysine aldehyde with a hydroxylysine residue in an adjacent molecule to form a Schiff base (fig. 1.7). This covalent linkage is an aldimine called dehydro-hydroxylysinonorleucine (dehydro-HLNL). Dehydro-HLNL can be reduced in vitro with KBH_4 to give HLNL (fig. 1.8). In the case of collagens where hydroxylation of N- and C-terminal lysine residues is extensive, the enzyme lysyl oxidase can also convert hydroxylysine to an aldehyde in the same way that it does lysine. The product of reaction of this hydroxylysine aldehyde and the hydroxylysine of the adjacent molecule is a dihydroxylated Schiff's base and is called dehydro-dihydroxylysinonorleucine. This compound undergoes spontaneous rearrangement through the Amadori mechanism to form an oxo-imine called hydroxylysino-5-oxo-norleucine (HLONL). HLONL can also be reduced *in vitro* to form dihydroxylysinonorleucine (DHLNL) (fig. 1.9).

The proportion of these two intermolecular reducible crosslinks is related to the degree of lysine hydroxylation. This varies between different tissues and also with development. For example, rat Achilles tendon contains equal proportions of each crosslink whilst rat tail tendons contain entirely the aldimine type of crosslink (Bailey & Light, 1989).

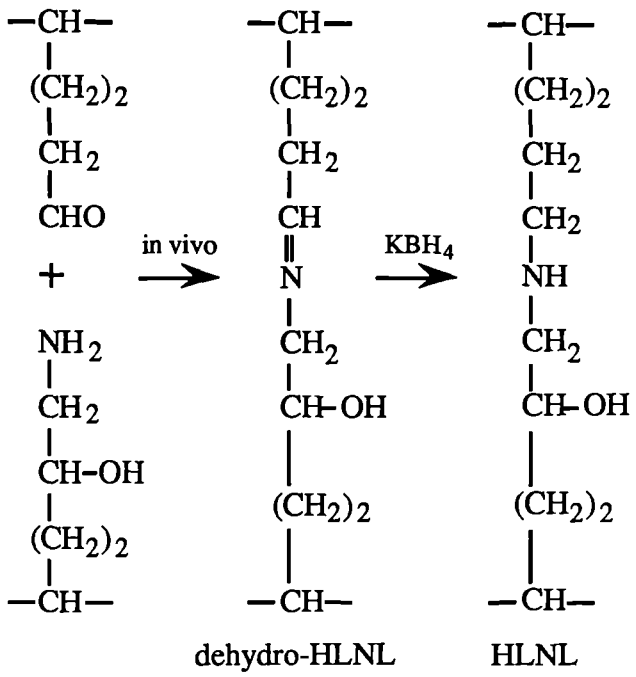


Fig. 1.8 The formation and reduction chemistry of the aldimine reducible crosslinks of collagen. (Bailey & Light, 1989)

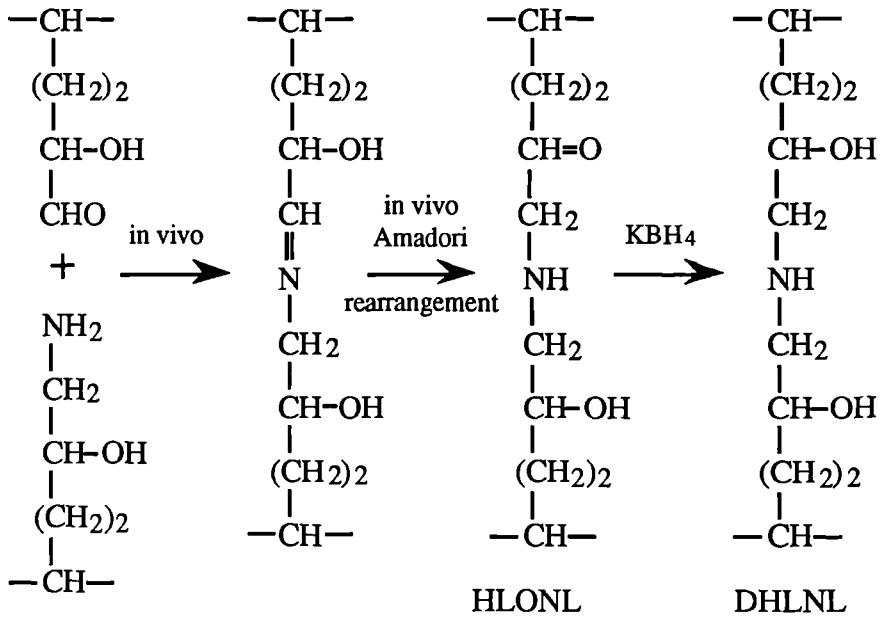


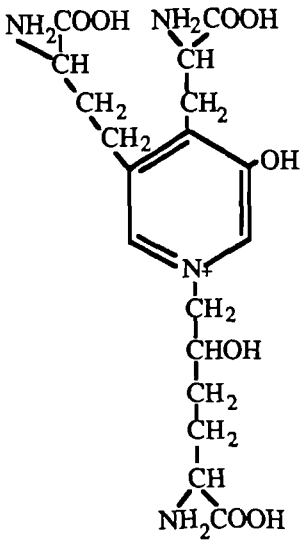
Fig. 1.9 The formation, rearrangement and reduction chemistry of the oxo-imine reducible crosslinks of collagen. (Bailey & Light, 1989)

Lysyl oxidase mediated mature crosslinks

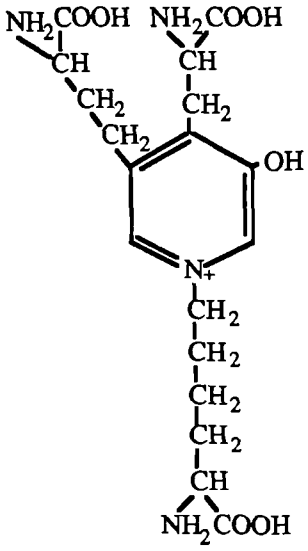
As collagen ages it undergoes progressive changes characterised by decreases in solubility (Schnider & Kohn, 1981 & 1982), elasticity (Aschner, 1960), increases in thermal stability (Snowden *et al.*, 1982) and resistance to enzymatic digestion (Hamlin *et al.*, 1978). This suggests an increase in the number of crosslinks. However, the crosslinks dehydro-HLNL and HLONL actually decrease with age. It was found that these crosslinks were not disappearing as such, but further reacting to form crosslinks with a higher valency, known as mature crosslinks.

Hydroxypyridinium crosslinks are mature, trifunctional, fluorescent crosslinks of which two analogues, hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP), have been reported (Eyre *et al.*, 1984). HP is formed by the reaction of HLONL with a hydroxylysine aldehyde residue while lysylpyridinoline is formed by the reaction of dehydro-HLNL and a hydroxylysine aldehyde residue (fig. 1.10).

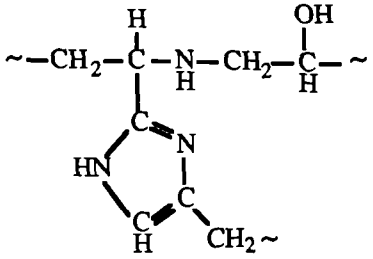
Mechanic and colleagues (1987) have shown that a further trifunctional crosslink is formed when dehydro-HLNL reacts with a histidine residue (fig. 1.10). This mature crosslink is called histidinohydroxylysinonorleucine or HHL (Mechanic *et al.*, 1987) and is the major known crosslink of mature skin. Various other compounds have been isolated from mature collagen and denoted as mature crosslinks but few have been characterised.



HP



LP



HHL

Fig. 1.10 The structures of some mature lysyl oxidase mediated crosslinks. HP (hydroxylysylpyridinoline), LP (lysylpyridinoline), HHL (histidinohydroxylysinonorleucine).

Non-enzymatically derived crosslinks

A third type of crosslink is derived from non-enzymatically glycosylated lysine and hydroxylysine residues. The maturational pathways of sugar derived crosslinks are far less well understood than those of the lysyl oxidase initiated crosslinks. The series of reactions is known as the Maillard reaction or non-enzymatic browning (Last *et al.*, 1990). Glucose is the sugar most commonly involved but fructose and a pentose can also participate. The first step is the condensation of a sugar aldehyde or ketone with a free amino group resulting in a Schiff base. The Schiff base is likely to undergo a rearrangement to form an Amadori product. It is believed that there are two main pathways by which advanced Maillard products are formed. Amadori products may

condense with each other to form imidazole Maillard products. Alternatively, the initial Amadori product may be degraded into a reactive alpha-dicarbonyl group. These new functional groups react with free amino groups to form pyrole-based pigmented and fluorescent adducts and crosslinks. These crosslinks, unlike lysyl oxidase mediated mature crosslinks, can be located between the helical regions of collagen molecules (fig. 1.11).

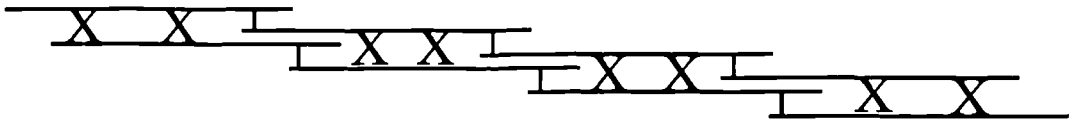
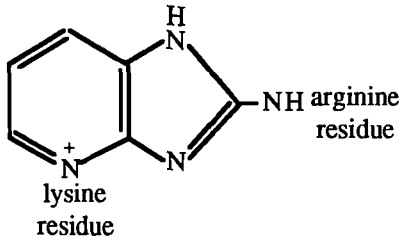


Fig. 1.11 Location of non-enzymatically derived crosslinks. (X)

Only one of these crosslinks has been characterised and has been given the name, pentosidine (Sell & Monnier, 1989). Pentosidine is formed from a pentose sugar a lysine residue and an arginine residue (fig. 1.12).



pentosidine

Fig. 1.12 The structure of pentosidine.

1.5.5 Packing of Collagen Molecules in Fibrils

Fibril conformation

Collagen fibrils can be visualised, and diameters measured in a transverse section, using electron microscopic techniques. Fibril diameters vary greatly, in some tissues, for example cornea, diameters are very regular (Maurice, 1984). In tendon however, fibril diameters range from 20 - 400 nm. Mature SDFT has been found to have a bimodal fibril distribution with the mean diameters of the two peaks being 35 and 215 nm (Parry *et al.*, 1978a). In some tissues, in longitudinal sections, a helical pattern has been observed within the collagen fibril (Wykoff, 1949; Reed *et al.*, 1956; Szirmai *et al.*, 1970; Bouteille & Pease, 1971; Rayns, 1974; Belton *et al.*, 1975; Lillie *et al.*, 1977). In tissues subjected to tension such as tendon however, collagen molecules run straight; a helical arrangement of the collagen fibril would result in some of the tensile load being converted into radial compression (Raspanti *et al.*, 1990).

Collagen type

Several tissues with different collagen fibril diameters and orientations and hence mechanical properties contain both type I and III collagen e.g. tendon, skin, aorta and amnion (Keene *et al.*, 1987). Type I and III collagen molecules show a remarkable homology to each other (Miller, 1985) and both form fibrils with the same periodic D-banding (Adachi & Hayashi, 1985). The difference in mechanical properties therefore, in tissues with different ratios of type I and III collagen is thought to be due to the way in which the two collagens form fibrils. Type III collagen is thought to form only small diameter fibrils due to the persistence of the amino propeptide while type I collagen forms larger diameter fibrils (Fleischmajer *et al.*, 1981). The arrangement of type I and III collagen in fibrils however is not completely understood. Studies by Keene and colleagues (1987) have found that fibrils in skin, tendon and amnion are copolymers of both type I and III collagen.

1.5.6 Collagen Fibres

Crimp

Collagen fibres are assembled from bundles of fibrils and have a diameter ranging from 1 - 10 μm. Fibres, which are visible under the light microscope, lie parallel to each other and run in the longitudinal axis of the tendon. Lying between the collagen fibres and also running parallel to them are rows of tendon cells.

Collagen fibres observed in most tissues are seen to be in some sort of waveform (Rigby *et al.*, 1959; Abrahams, 1967; Viidik & Ekholm, 1968), referred to as crimp (fig. 1.13) (Diamant *et al.*, 1972). The crimp results in the birefringent nature of tendon tissue which, when viewed under a polarising microscope, appears as a series of light and dark bands (fig. 1.14). The wave structure was found to be a planar zig-zag (Diamant *et al.*, 1972) and is clearly visible at the level of banded fibrils. The crimp is extremely sharp and is confined to within 1- to 2-D periods (Dlugosz *et al.*, 1978). The initiation and further development of crimp remains unclear. It has been suggested that a passive mechanical mechanism is responsible, where shrinkage of the matrix results in fibre buckling (Dale & Baer, 1974; Viidik, 1980). Alternatively, fibroblasts, which are well supplied with contractile proteins, may participate in an active contraction of the matrix (Shah *et al.*, 1982).

Gross structure

A collection of fibres enclosed in a fine membrane of connective tissue, the endotenon, is referred to as a primary fibre bundle or subfascicular unit. A fascicle is a group of 3-20 subfascicular units with a diameter of about 600μm which is surrounded by another sheath the epitenon. Finally a few fascicles form a gross tendon (fig. 1.15).

The cross section of the equine SDFT in the mid-metacarpal region is a comma shape and has an area of approximately 100 mm^2 (Wilson, 1991).

1.6 Blood Supply

The blood supply to the tendon is of great importance to supply nutrients and oxygen to the tissue and to remove carbon dioxide and lactic acid (Celli *et al.*, 1976; Potenza, 1976; Vailas *et al.*, 1978; Landi *et al.*, 1980 a,b & c), and was first described by Mayer (1916). Contrast media mixed with dye has been used to demonstrate that vessels from the paratenon supply the tendon at the muscle-tendon junction, whilst vessels from the periosteum supply the tendon at the site of insertion. Intratendinous

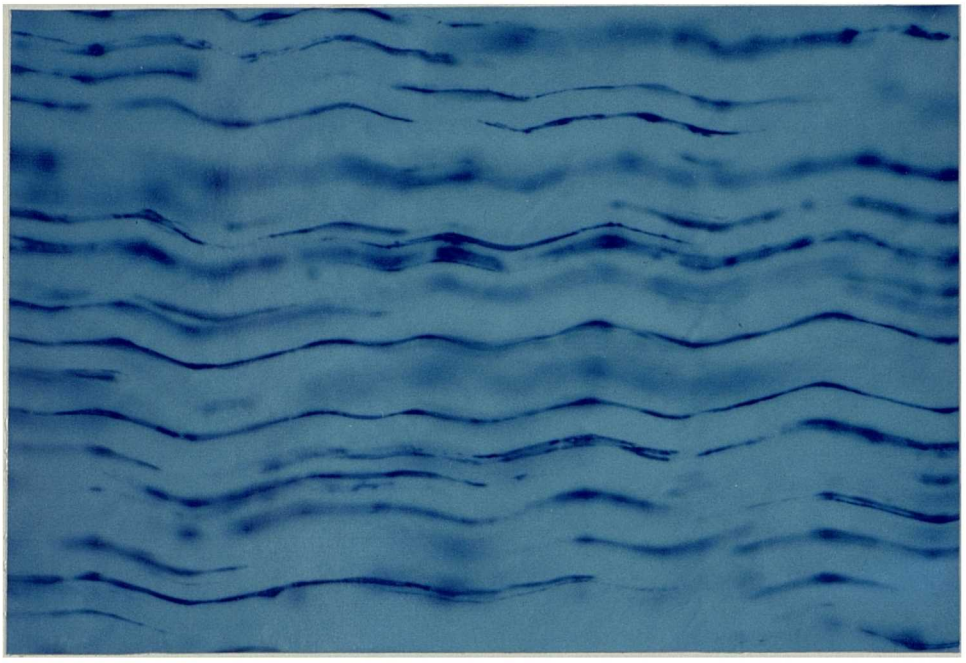


Fig. 1.13 Longitudinal section of equine superficial digital flexor tendon showing cells, stained for malate dehydrogenase activity with tetrazolium salts, following the direction of the crimped collagen fibres. (x 90)

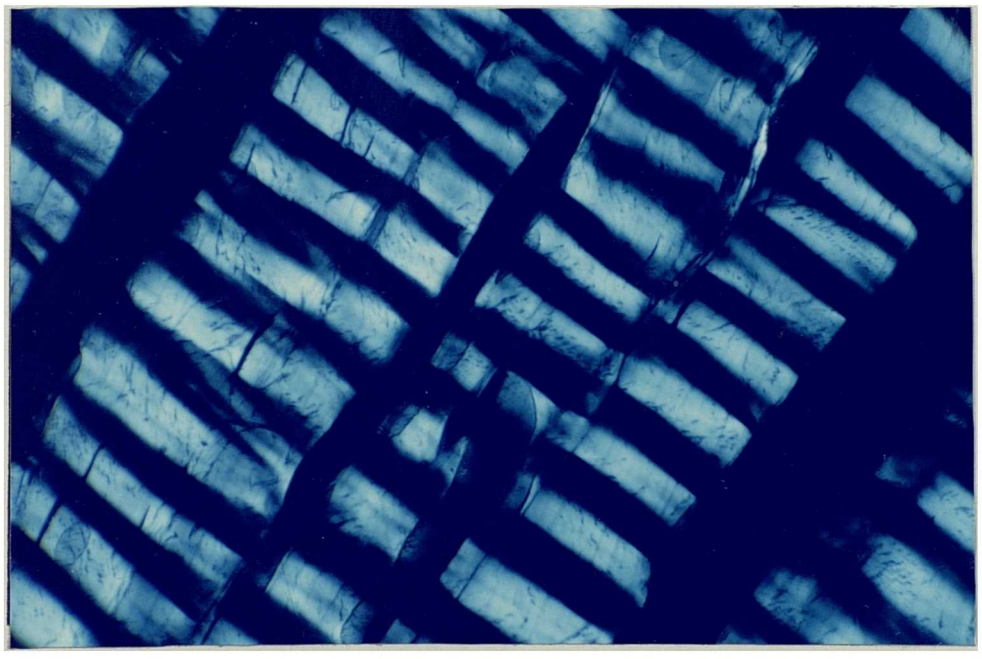


Fig. 1.14 Collagen fibres viewed under a polarising microscope showing light and dark banding pattern resulting from the crimped structure. (x 140)

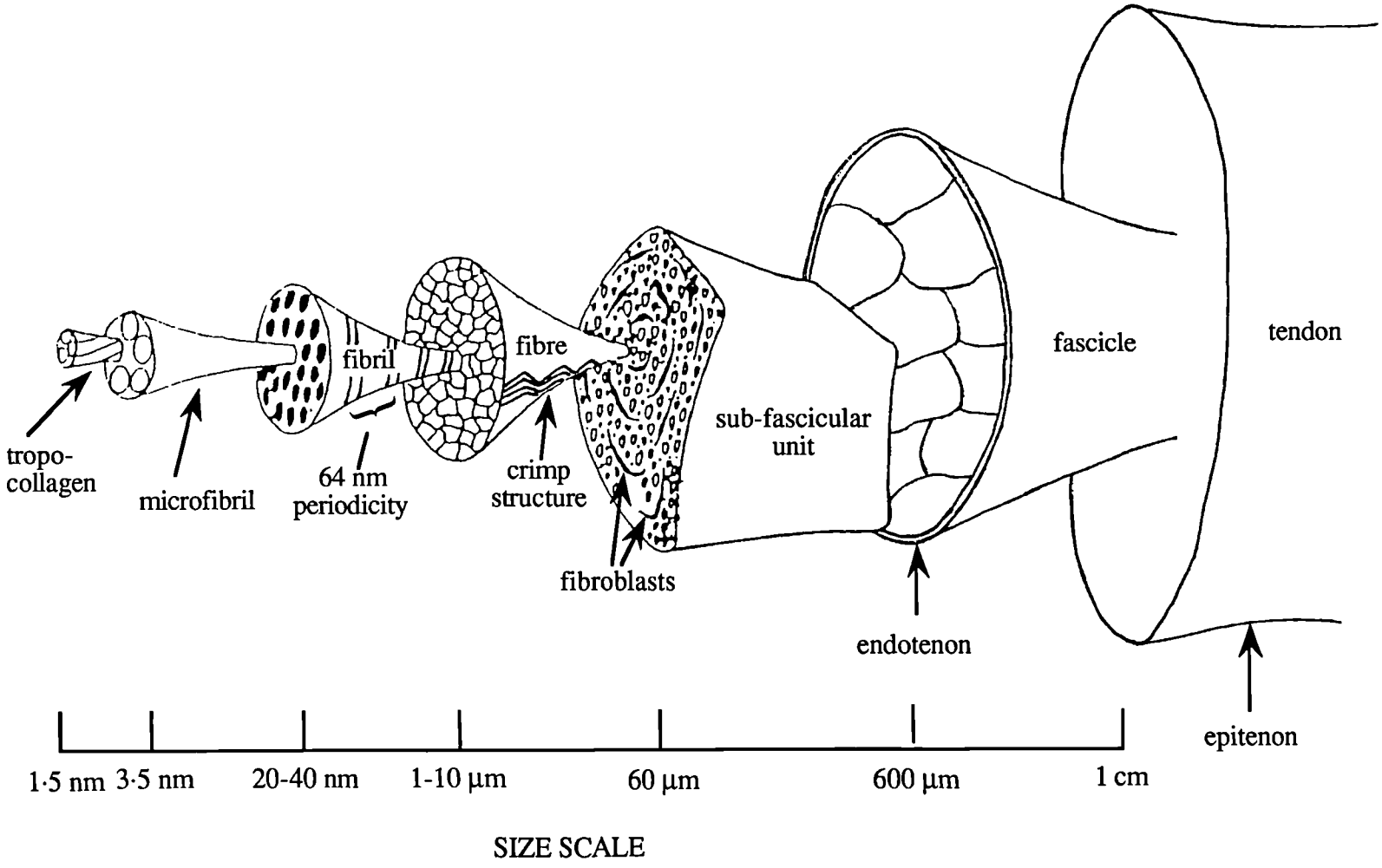


Fig. 1.15 Tendon hierarchy (modified from Kastelic *et al.*, 1978)

vessels were found to run longitudinally between the subfascicular units (Edwards, 1946) communicating with transverse vessels on the surface of the tendon to form a fine capillary network in the endotenon around the subfascicular units (Brockis, 1953; Smith, 1965). The mid-portion of long tendons was found to be supplied mainly by vessels from the surrounding paratendinous tissue (Peacock, 1959; Bergljung, 1968).

The vascular supply to the SDFT in the horse has been investigated by Strömberg & Tufvesson (1969) and Strömberg (1971). The vasculature of the tendon comprised of slender longitudinal vessels with very few transverse connections. The proximal segment of the tendon was supplied by some of the numerous perimysial vessels and also by vessels from the mesotenon of the carpal tendon sheath. The distal part of the tendon was supplied by vessels entering at the proximal and distal reflections of the digital tendon sheath. The mid-part of the tendon, where fewer vessels were seen, was supplied by branches from two fairly large arteries entering the tendon medially and laterally at the distal reflection of the carpal sheath (Strömberg, 1971).

The flow of blood through tendons has also been measured in several studies using washout techniques. Strömberg (1971) obtained a value of 1·08 ± 0·42 ml/100g/min. for the equine SDFT. Rates of 0·5 - 2·8 ml/100g/min. have been recorded for blood flow in other tendons from dogs, chickens and rabbits (Gross, quoted by Vailas *et al.*, 1978; Piaggi & Mingione, 1981; Landi *et al.*, 1983). Whether blood flow rates of this magnitude are sufficient to support normal cellular function in tendons is not clear. These values are similar to measurements of blood flow through resting human skeletal muscle (2·2 ± 0·65 ml/100g/min). However, maximal blood flow through human skeletal muscle was 54·9 ± 11·6 ml/100g/min (Lassen *et al.*, 1964).

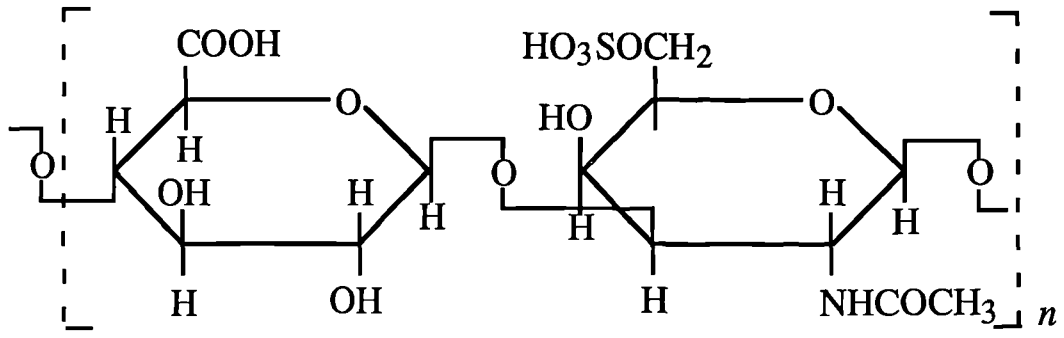
Maximal blood flow through equine SDFT (Strömberg, 1971), measured following application and removal of local circulatory arrest, was 160·2% ± 70·2 of the resting value. However, exercise alone produced no significant increase in blood flow in this tendon, whilst in rabbit tendons, exercise resulted in a stimulation of blood flow (~ 140%) above the resting value as measured using a washout technique (Landi *et al.*, 1983). Hooper *et al.* (1984) measured blood flow to tendons using two techniques; a radiolabelled microsphere technique and a washout technique. Clearance was significantly greater than flow in all tendons, indicating that diffusion from surrounding tissue maybe important. Naito & Ogata (1983), using a washout technique, found that dissection of the surrounding soft tissue from the Achilles tendon of adult rabbits caused a 35% reduction in blood flow. These results may also explain those of Landi; stretching the tendon during exercise could well increase diffusion out of the tendon rather than exercise resulting in a metabolically driven stimulation of blood flow.

1.7 Proteoglycans

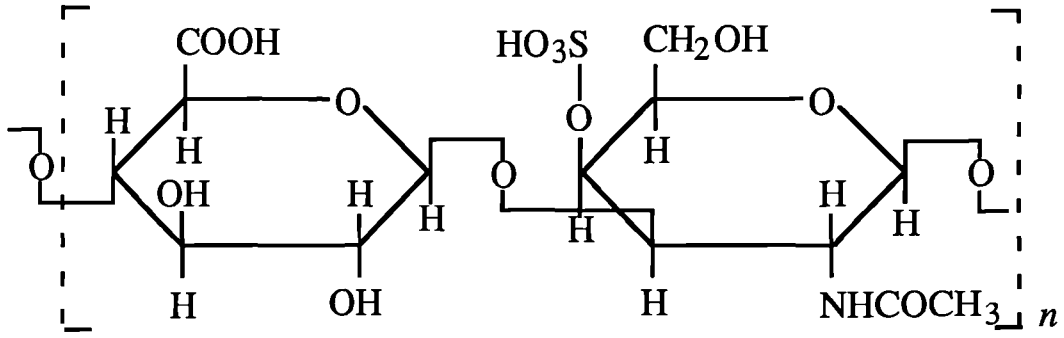
Proteoglycans are complex macromolecules that consist of a core protein to which one or more glycosaminoglycan (GAG) side chains attach. Differences in proteoglycan structure can result from differential expression of genes encoding their core protein as well as from variation in the number, length and types of GAG chain attached during the post translational modifications of the core proteins (Goetinck, 1991). GAG chains are polymers of repeating disaccharides and they may consist of chondroitin sulphate (glucuronic acid + N-acetylgalactosamine, sulphated in either the 4 or 6 position), dermatan sulphate (iduronic acid + N-acetylgalactosamine-4-sulphate), keratan sulphate (N-acetylglucosamine-6-sulphate + galactose), heparan sulphate or heparin (N-acetylglucosamine-6-sulphate + iduronic acid) and hyaluronic acid (N-acetylglucosamine + glucuronic acid) (fig. 1.16 & 1.17). The attachment of the GAG chain is through a linkage region which consists of xylose, galactose, galactose and a uronic acid residue followed by the repeating disaccharide units that make up the GAG chain proper. Several sequences involving serine residues have been reported as attachment sites of xylose by xylosyltransferase (Bourdon *et al.*, 1987; Doege *et al.*, 1987; Huber *et al.*, 1988; Zimmerman & Ruoslahtil, 1989).

The proteoglycan content of tendon has been studied in flexor tendons from various species. Rabbit flexor tendon was found to contain less than 0·2% proteoglycan of the dry wt; the predominant GAG side chain being dermatan sulphate. In the same tendon however, in the region passing over the joint where the tendon is subjected to compressional forces in addition to tension, proteoglycan formed 3·5% of the dry wt; the predominant GAG here being chondroitin sulphate (Gillard *et al.*, 1977). Similar observations have been made in adult bovine flexor tendon. In the tensional region of this tendon 12% of proteoglycans had a large protein core and chondroitin sulphate side chain while 88% had a much smaller protein core and dermatan sulphate side chain. The proximal pressure bearing region had a greater amount of large proteoglycans (Vogel & Heinegard, 1985), and at least a 500 fold increase in the amount of keratan sulphate (Vogel & Thonar, 1988). The difference in proteoglycan content is reflected by synthesis *in vitro* by explants of tissue from each of the two regions (Vogel *et al.*, 1986; Koob & Vogel, 1987b). Furthermore, alteration of the mechanical environment *in vivo,* by translocation of rabbit flexor tendon, resulted in a change in GAG synthesis to that characteristic of the new mechanical role (Gillard *et al.*, 1979).

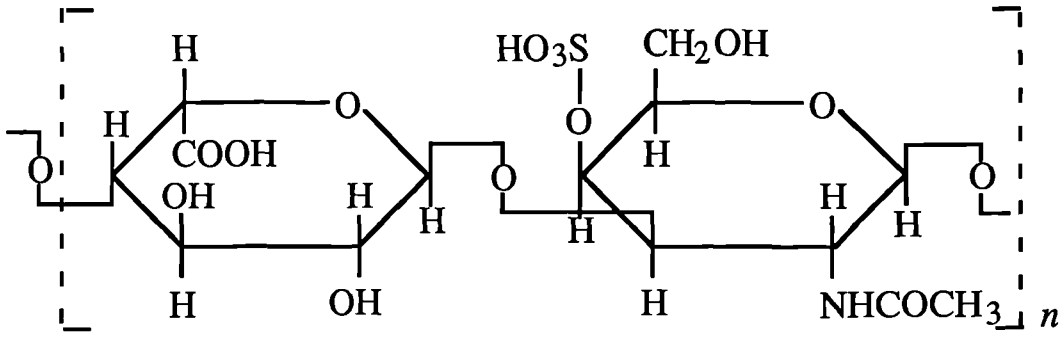
Cupromeronic blue dye enables proteoglycans to be visualised as electron dense filaments. In most tissues two types are present; a small thin collagen fibril associated filament and a thick heavily stained filament localised between bundles of collagen



chondroitin-6-sulphate

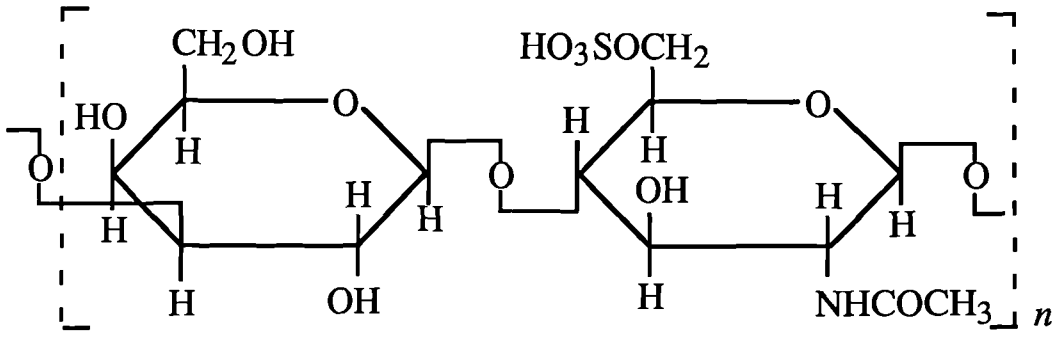


chondroitin-4-sulphate

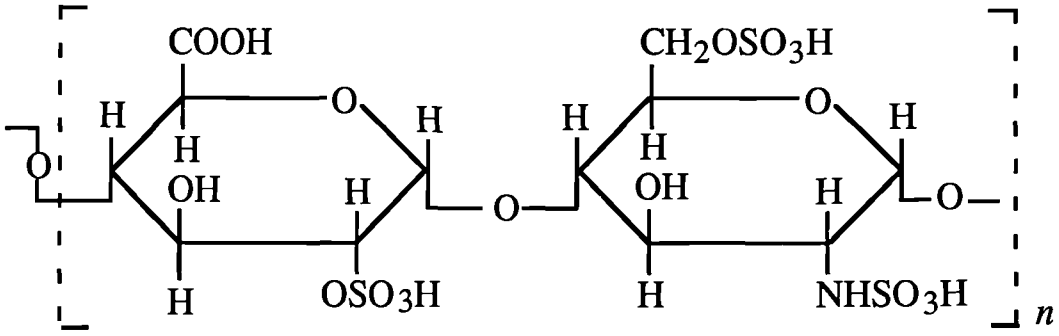


dermatan sulphate

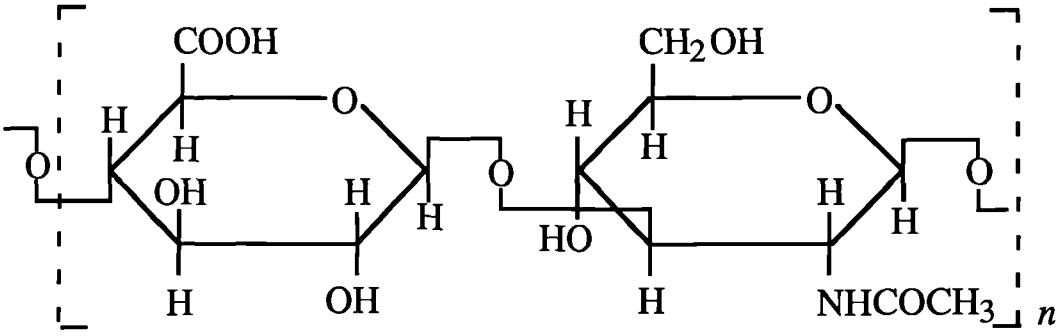
Fig. 1.16 Structure of the repeating disaccharide unit of chondroitin-6-sulphate, chondroitin-4-sulphate and dermatan sulphate.



keratan sulphate



heparan sulphate



hyaluronic acid

Fig. 1.17 Structure of the repeating disaccharide unit of keratan sulphate, heparan sulphate and hyaluronic acid.

fibrils (Van Kuppevelt *et al.*, 1987). The small thin filament of proteoglycan is likely to contain the dermatan sulphate GAG sidechain, as dermatan sulphate is specifically, regularly and entirely associated with collagen fibrils (Scott, 1984). The specific binding site for dermatan sulphate is the d band (Scott & Haigh, 1985), close to the initial site of calcification of type I fibrils (fig. 1.18). It has therefore been suggested that dermatan sulphate might play a role in preventing soft connective tissues from calcifying. The interaction of dermatan sulphate with the collagen fibril occurs via the core protein (PG II) of the proteoglycan (Vogel *et al.*, 1987; Brown & Vogel, 1989). Later studies (Scott, 1991) have shown that dermatan sulphate proteoglycan binds to the collagen fibril at both the d and e band, whilst keratan sulphate proteoglycan, also fibril associated, binds at the a and c band. Both proteoglycans give rise to the one proteoglycan, one binding site, hypothesis (Scott, 1991). Hyaluronate and chondroitin sulphate proteoglycan are probably mainly interfibrillar, acting in a space filling capacity (Scott, 1986), the latter giving rise to the thick, heavily stained filament localised between bundles of collagen fibrils.

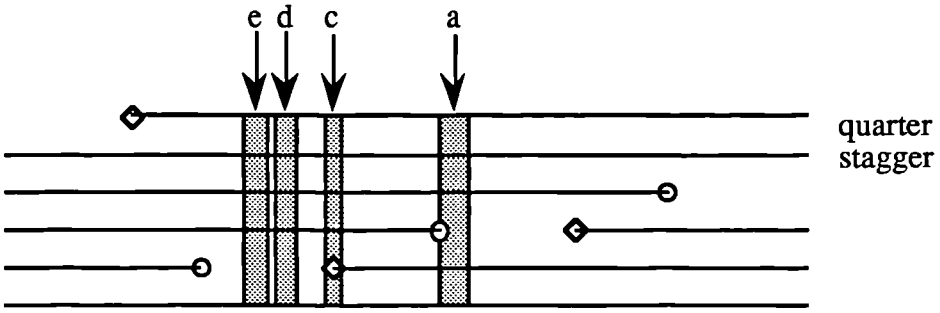


Fig. 1.18 Proteoglycan binding sites shown against the arrangement of collagen molecules in quarter-stagger. ◇, N-terminal; O, C-terminal. (Scott, 1988)

Proteoglycans are thought to play a rôle in fibrillogenesis and control of fibril diameters (Scott, 1986). In favour of this view, increase in fibril diameter during development coincides with a decrease in hyaluronate and chondroitin sulphate content (Scott & Hughes, 1986). The large amount of proteoglycan and hyaluronate in young tissues might help to keep the protofibrils and subfibrils from coalescing, thereby allowing rapid access of reactants to the interior of thick fibrils (Scott, 1990). This mechanism might also be important during tissue remodelling. Further evidence for a rôle of proteoglycans in control of fibril diameters comes from an *in vitro* study, where dermatan sulphate was found to be able to inhibit fibrillogenesis of bovine type I and II collagen, the reaction depending on the protein core (Vogel *et al.*, 1984).

1.8 Connective Tissue Glycoproteins

It became clear in the early 1960's that besides collagen, elastin and proteoglycans there were another family of macromolecules that did not fit into any of these groups. These are termed structural or connective tissue glycoproteins (Labat-Robert, 1990). The best characterised are fibronectin, a glycoprotein distributed throughout most connective tissues, and laminin which is restricted to basement membranes. Other glycoproteins include tenasin, thrombospondin, von-Willibrand factor, nidogen and fibrillin.

Fibronectin is able to bind to collagen and has a particularly high affinity for denatured collagens (Jilek & Hormann, 1978). Fibronectin has also been shown to interact with specific cell membrane receptors (integrins). Integrins are capable of transmitting signals from the extracellular matrix to the interior of the cell (Ruoslahti & Pierschbacher, 1987; Ruoslahti & Giancotti, 1989). Therefore fibronectin can serve as a mediator between the collagen matrix and the interior of the cell.

1.9.1 Tendon Cells

Tendon fibroblasts are responsible for the synthesis, assembly, deposition and organisation of extracellular matrix molecules and thus determine the morphology of the tissue (Doane & Birk, 1991). Fibroblasts in tendons are orientated parallel to each other and form long rows between collagen fibres. Cell orientation is important in determining how the matrix is assembled and orientated (Goodship *et al.*, 1980). The matrix in turn can feedback information to the cell and alter the programme of synthesis (fig. 1.19). Little is known, however, about the receptors and transmission of signals to the cell interior (Labat-Robert *et al.*, 1990). One possibility is that mechanical deformation of the cell membrane may activate stretch-activated ion channels (Guharay & Sachs, 1984). Alternatively, it has been suggested that cell shape correlates with matrix synthesis (von der Mark *et al.*, 1977; Benya & Schaffer, 1982; Zanetti & Solursh, 1984). Mechanical forces may also alter the vascular supply and nutrient flow through the tissue and modulation of the synthetic programme of tendon fibroblasts may follow from something as simple as a change in oxygen supply (Bassett & Herrmann, 1961).

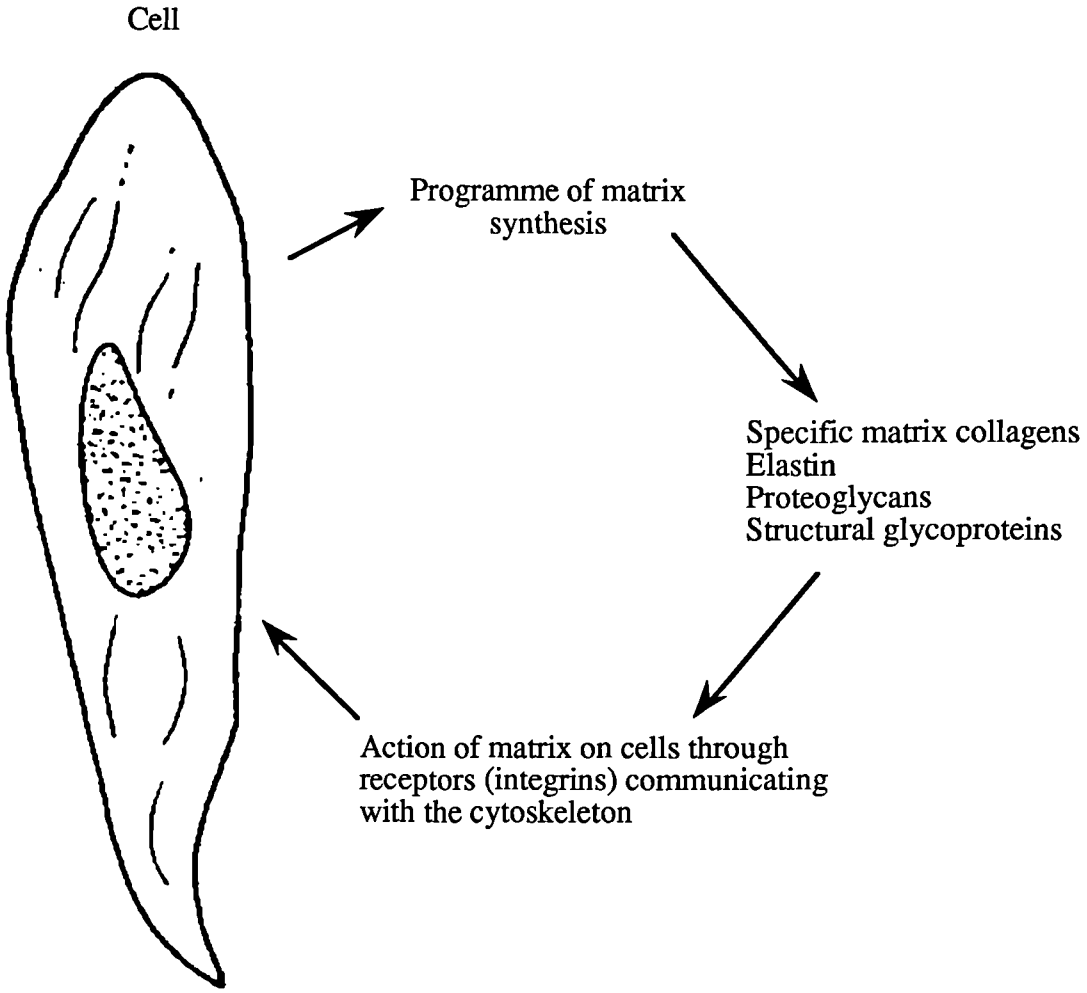


Fig. 1.19 The informational feedback loop between the cell and the extracellular matrix. (Labat-Robert, 1990)

1.9.2 Collagen Metabolism

Both synthesis and degradation of collagen are important in collagen metabolism. The synthesis of collagen by fibroblasts has been discussed in section 1.5.2. Fibroblasts have also been shown to synthesise and secrete a group of proteinases that are able to degrade collagen under physiological conditions. These proteases can be divided into four classes: aspartic, cysteine, metallo- and serine active sites.

Collagenase, the best characterised protease, is a Zn^{2+}-metallo-endopeptidase secreted from the cell in an inactive procollagenase form. Activation is achieved by proteolysis of the extension peptide. Once activated, collagenase binds to the extracellular collagen fibril cleaving the collagen molecule across all three α chains to give two triple helical fragments (Sakai & Gross, 1967). Where collagen molecules are stabilised by intermolecular crosslinks, the triple helical fragments, formed by the action of collagenase, are not released from the fibre since both fragments are still attached to adjacent molecules through their crosslinks. Release from the fibre can only be achieved by the action of other enzymes on the non-helical region around the crosslink residue. Cysteine, aspartic and serine proteases are able to act at neutral pH on the non-helical end regions of the molecule (Rubin *et al.*, 1963) between the cross-link and the triple helix thereby releasing collagen molecules and the triple helical fragments formed by collagenase action from the fibre. Following release, collagen molecules and fragments are denatured at 37°C and then rapidly attacked by most proteinases.

Digestion of fragments released by the neutral collagenase and proteinases can occur following endocytosis. Fragments are taken up by cells in vacuoles which then fuse with phagolysosomes. Within the lysomes collagen fragments may then be completely hydrolysed by enzymes such as cathepsins B, L and N (Jackson, 1973). The optimal activity of these lysosomal cysteine proteinases is at around pH 3·5 - 4·0 and therefore their activity is expected to be confined to within the lysosome.

1.9.3 Energy Metabolism

All processes involved in growth and metabolism of cells require an input of energy. In most cases this energy is supplied by hydrolysis of high-energy phosphate bonds in adenosine triphosphate (ATP) and thus cells must be able to generate ATP. In animal cells, the most important fuels for generation of ATP are sugars such as glucose, and fatty acids.

Glycolysis is the sequence of reactions that converts glucose into pyruvate with the concomitant production of ATP (Stryer, 1988) (fig. 1.20). The reactions of this pathway take place in the cell cytosol and are nearly universal in biological systems. The net reaction in the transformation of glucose into pyruvate is

$$\text{Glucose} + 2\,P_i + 2\,ADP + 2\,NAD^+ \longrightarrow$$
$$2\,\text{pyruvate} + 2\,ATP + 2\,NADH + 2\,H^+ + 2\,H_2O$$

Thus, two molecules of ATP are generated in the transformation of glucose into pyruvate.

The fate of pyruvate in the generation of metabolic energy is variable. In the absence of oxygen pyruvate can be reduced by nicotinamide adenine dinucleotide (NADH) to form lactate catalysed by the enzyme lactate dehydrogenase. In the conversion of glucose to lactate there is no net oxidation-reduction. The NADH formed in the oxidation of glyceraldehyde 3-phosphate is consumed in the reduction of pyruvate, thus regenerating NAD^+ and enabling glycolysis to be maintained. Only a small fraction of energy, however, is released from glucose in its anaerobic conversion to lactate.

Under aerobic conditions pyruvate enters the mitochondria where it undergoes oxidative decarboxylation to form acetyl coenzyme A (acetyl CoA). The two carbon acetyl unit enters the citric acid cycle, also known as the Krebs cycle (fig. 1.21), by condensation with oxaloacetate. The two carbon atoms leave the cycle in the form of CO_2 in the successive decarboxylations catalysed by isocitrate dehydrogenase and 2-oxo-glutarate dehydrogenase. Two NAD^+ molecules are reduced in the above reactions, one flavin adenine dinucleotide (FAD) molecule is reduced in the oxidation of succinate , and one NAD^+ molecule is reduced in the oxidation of malate. The net reaction of the citric acid cycle is:

$$\text{Acetyl CoA} + 3\,NAD^+ + FAD + GDP + P_i + 2\,H_2O \longrightarrow$$
$$2\,CO_2 + 3\,NADH + FADH_2 + GTP + 2\,H^+ + CoA$$

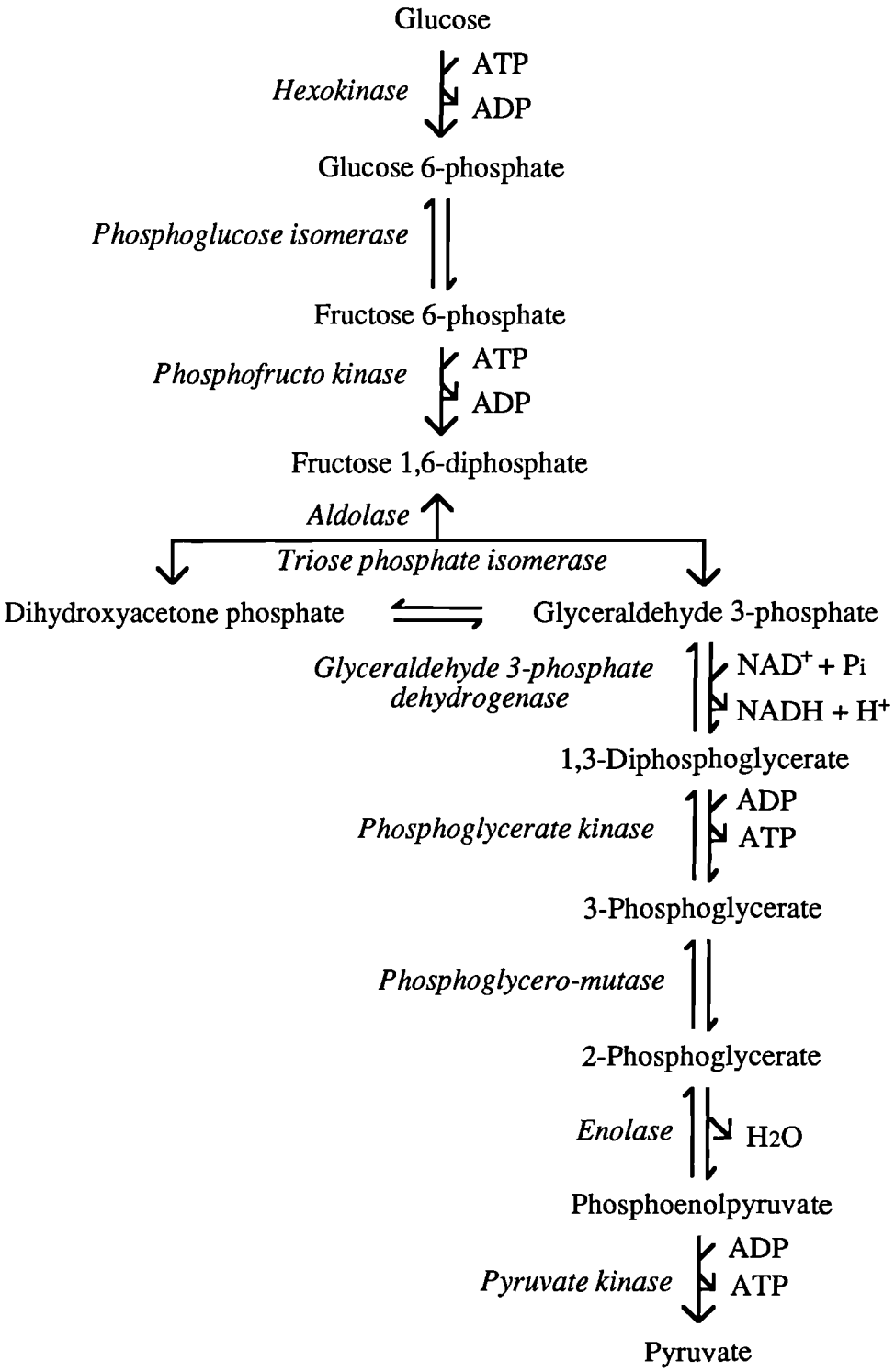


Fig. 1.20 The glycolytic pathway.

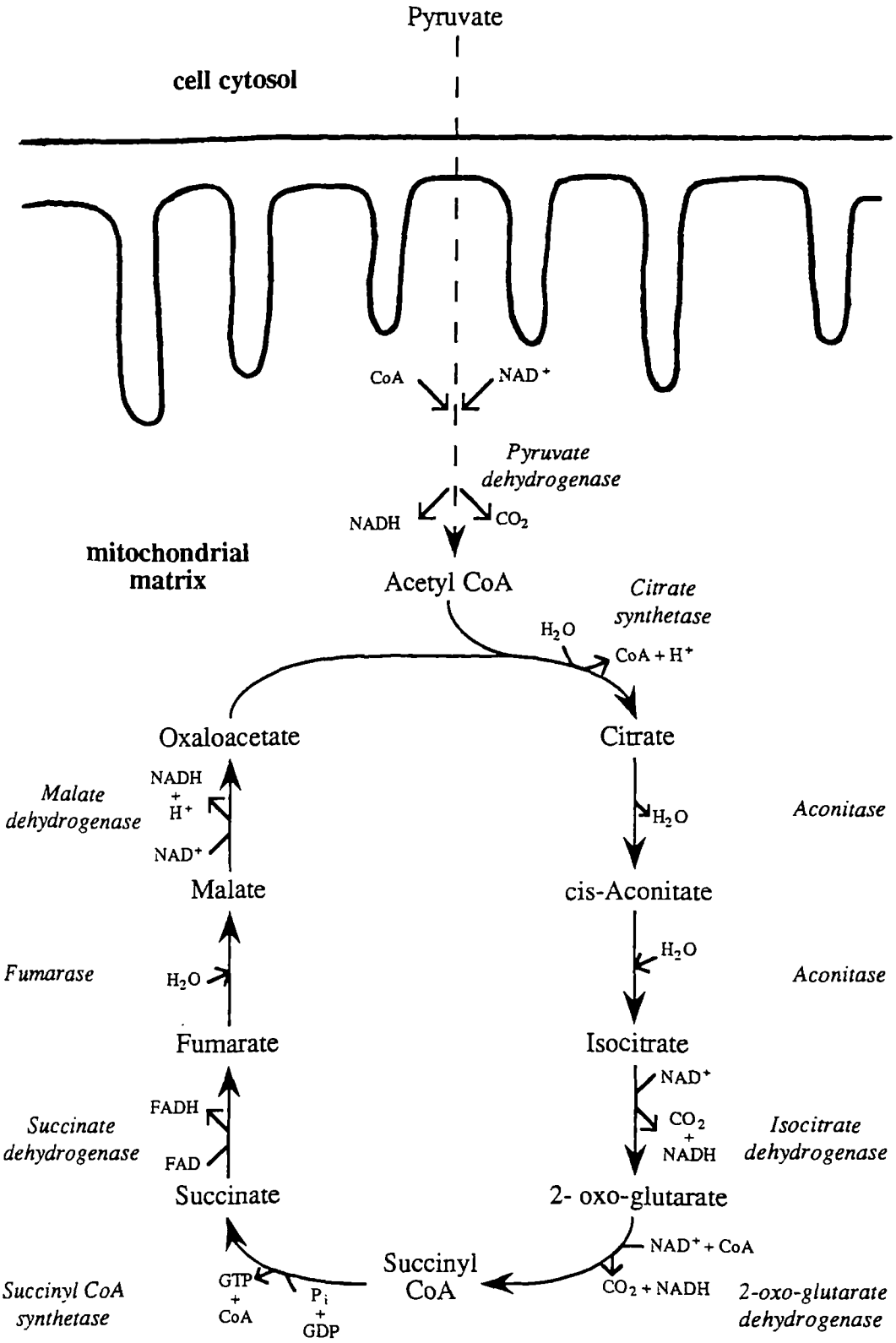


Fig. 1.21 The citric acid cycle

Only one high-energy phosphate bond per acetyl unit is directly formed in the citric acid cycle. Most of the ATP is formed during the oxidation of NADH and $FADH_2$ by the electron transport chain. Three ATPs are formed for each NADH in the mitochondrion, whereas two ATPs are generated per $FADH_2$. The citric acid cycle only operates under aerobic conditions, even though oxygen does not participate directly. This is because NAD^+ and FAD can be regenerated in the mitochondrion only by electron transfer to molecular oxygen.

The step-by-step transfer of electrons from NADH and $FADH_2$ to O_2 occurs through numerous electron carriers such as cytochromes, and leads to the pumping of protons out of the mitochondrial matrix and to the generation of a membrane potential across the mitochondrial inner membrane. ATP is synthesised when protons flow back to the mitochondrial matrix through an enzyme complex. Thus, oxidation and phosphorylation are coupled by a proton gradient across the inner mitochondrial membrane.

The complete oxidation of one molecule of glucose to CO_2 and H_2O forms 36 molecules of ATP, while the conversion of glucose to lactate forms only 2 ATP molecules.

Energy metabolism in tenocytes

Whether mature tendon cells are capable of oxidative energy metabolism is a question which has not been clearly resolved. A number of studies on rat tendons have suggested that collagen turnover in this structure is essentially zero in the mature animal (Neuberger *et al.*, 1951; Thompson & Ballou, 1956) and implied that tendon cells might be metabolically inert. More recent studies have used as indices of oxidative metabolism the presence of mitochondrial enzyme activities. Thus, Landi *et al.* (1980 a) suggested that the mitochondrial enzyme, succinate dehydrogenase (SDH) which is measurable in young rabbit tendon, is absent in the adult tendon. Consistent with this, Floridi *et al.* (1981) demonstrated that mature rabbit tendon is apparently incapable both of oxidative and anaerobic utilisation of glucose, again supporting the notion of metabolic inertness of the tendon in this species. These data, however, contrast with the demonstration of cytochrome oxidase (CytO) activity in extracts of mature *Galago Senegalensis* (bush baby) Achilles tendon (Tipton *et al.*, 1979), and NAD^+-linked isocitrate dehydrogenase (ICDH-NAD^+, an exclusively mitochondrial enzyme) in mouse Achilles tendon homogenates (Heikkinen *et al.*, 1975). Furthermore, Józsa *et al.* (1979) have shown histochemically that human Achilles tendon possesses both SDH and CytO activities, and provided electron micrographic evidence for the presence of mitochondria within tenocytes in tissue sections. Further, studies on human tendon

have indicated that this mature tendon has a small, but measurable, oxygen consumption (Peacock, 1957).

1.10 Tendon Function

The principle rôle of tendon is to transmit the force generated when a muscle contracts into the movement of a joint (Evans & Barbenel, 1975). In the 16th century, Leonardo da Vinci first described the tendon in "Quarderni d'Anatomia" as being a mechanical instrument which carries out as much work as is entrusted to it. However, whilst this passive rôle has long been recognised, in recent years other mechanical functions have been attributed tendon.

One of these functions is to act as a protective mechanism during rapid and unexpected movement (Smith, 1954; Barnett *et al.*, 1961). This is because the compliance of a tendon enables it to act as a mechanical buffer to protect muscle fibres from damage during eccentric contractions (Griffiths, 1991). In the case of the SDF musculotendinous unit, however, it is the accessory ligament which is responsible for transferring load away from the muscle belly (Shoemaker *et al.*, 1991).

Tendons can also function as dynamic amplifiers (Hill, 1951), rather like a catapult mechanism increasing the *power* output of the muscle. Insects use elastic structures in this way enabling them to jump much higher than they would otherwise be able (Bennet-Clarke, 1976). However, the vestigial nature of the SDF muscle means that the musculotendinous unit is unlikely to function in this manner.

Tendons can also act as elastic energy stores (Dawson & Taylor, 1973). This provides an important mechanism for increasing the *efficiency* of muscular locomotion and hence a means of saving substantial quantities of muscular energy (Alexander, 1984 & 1988; Cavagna *et al.*, 1977). Energy is stored transiently as strain in tendons that are stretched by impact forces as the foot hits the ground. Elastic recoil reconverts most of this stored energy into kinetic forms as the foot leaves the ground (Alexander, 1983). Metabolic energy savings provided by this tendon elasticity during fast locomotion of large animals may be as high as 50% (Alexander, 1984 & 1988; Cavagna *et al.*, 1977). This may be a major function of the SDFT for the following reasons. Firstly, digital flexor tendons are of a design well suited to enable them to act as such elastic energy stores (Shadwick, 1990), and secondly the horse has evolved for efficient high speed locomotion.

1.11.1 Biomechanical Behaviour

The physical characteristics of tendon include great tensile strength, flexibility, considerable inextensibility and almost perfect elasticity (Elliott, 1965). The tensile strength was the first of these to be studied (Valentin *et al.*, 1844).

Tendons do not obey Hooke's law, and as long ago as 1847, the stress strain curve was likened by Wertheim to a hyperbola whose apex is at the origin of the co-ordinates. The stress-strain behaviour is more commonly reported as comprising an initial lax response, with progressive stiffening, leading to a quasi-linear relationship. However, at even higher stresses the tissue "yields" and so the entire stress-strain relationship is best described as sigmoidal (fig. 1.22) (Evans & Barbenel, 1975). The basic form of the quasi-static stress-strain relation for tendon was established some fifty five years ago (Gratz & Blackberg, 1935).

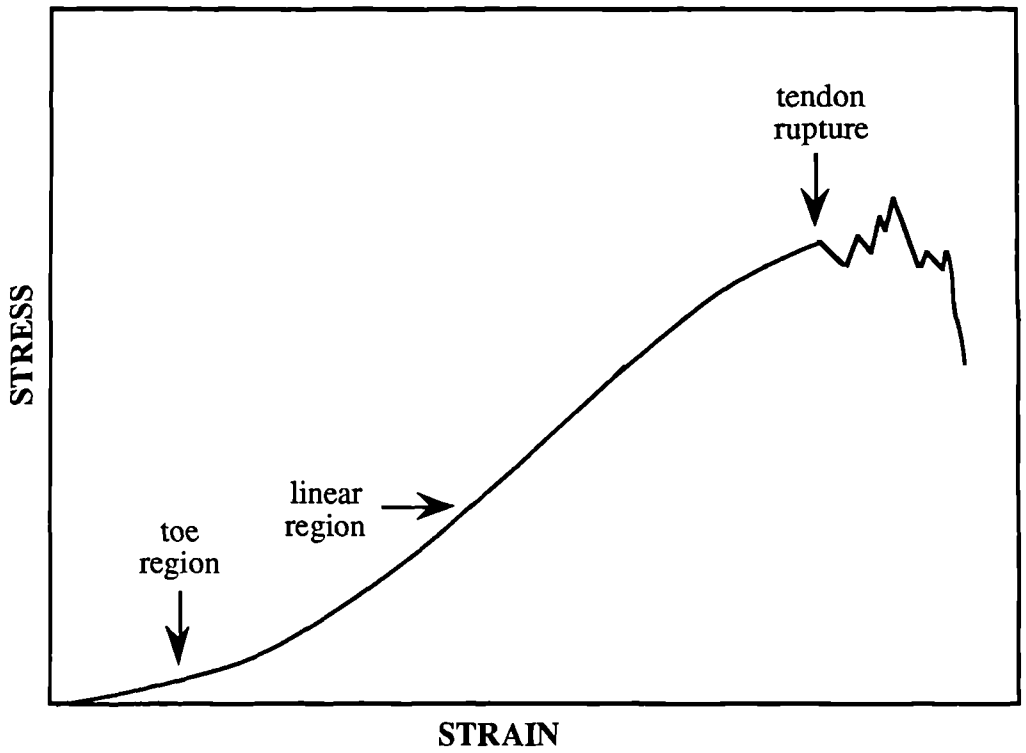


Fig. 1.22 Diagrammatic representation of a stress-strain curve for loading of a tendon.

In the initial concave portion of the curve little force is required to elongate the tissue. This part of the curve has been termed the "toe" region and is due to the straightening out of the crimped collagen fibres. In this region tendon is elastic; unloading restores both the wave pattern and the reference length (Butler *et al.*, 1978). A gradual transition occurs typically at about 3% extension (Viidik, 1968) when the

tendon becomes much more resistant to elongation. This transition from the initial lax phase corresponds with the disappearance of the waveform on the surface of the tendon (Rigby *et al.*, 1959; Strömberg & Wiederhielm, 1969; and Viidik, 1968 & 1972).

The second part of the curve shows a linear response in elongation to an increase in load. At this point in loading there is a uniform stretching of the triple helices of the collagen molecule itself and also a stretching of the telopeptides resulting in an increase in the D period (Mosler *et al.*, 1985). On unloading a proportion of the energy is not returned, but is dissipated, mostly as heat (Harkness, 1979). For mammalian tendons about 8 - 13% of energy is lost in this way (Olmos *et al.*, 1989).

The linear loading endpoint signifies the first failure of fibre bundles. Once the maximum load is attained the complete failure then occurs rapidly. Steven & Minns (1975) suggest that rupture occurs at a molecular level; the collagen molecule itself giving way. This is not generally accepted however, slippage of microfibrils and tropocollagen units are probably the prime mechanism of yielding (Kastelic & Baer, 1980).

The mechanical properties of equine SDFT have been tested *in vitro* (Wilson, 1991) and values of 19·7% ± 3·9 for ultimate strain and 13·6 KN for ultimate force obtained. Using the figure of 100 mm^3 for SDFT cross sectional area (Wilson, 1991) this gives a predicted ultimate stress of 136 MPa for equine SDFT.

During the gallop strains of 12 - 16% are reached in the equine forelimb SDFT (Herrick *et al.*, 1978, Stephens *et al.*, 1989), suggesting that this tendon is working near to its mechanical limits.

1.11.2 Effect of Matrix Composition on Biomechanical Properties

The tensile strength of a tendon cannot simply be predicted from the cross sectional area with no regard for matrix composition (Riemersima & Schamhardt, 1985). Biomechanical properties will not only be affected by the amount of collagen present but also the way in which it is arranged and crosslinked together. For example, towards the end of pregnancy there is a fall in the tensile strength of the foetal membranes although the amount of collagen per unit area remains unchanged (Harkness & Harkness, 1956).

Some components of the extracellular matrix have been directly related to their biomechanical influence on the tissue. Large diameter collagen fibrils have a greater tensile strength than an equivalent mass of collagen arranged in small fibrils (Parry *et al.*, 1978b). In large fibrils the potential density of intrafibrillar crosslinks is increased. However, in tissues subjected to high strains, the ability to withstand creep is important. In such tissues fibrillar diameter is decreased, thereby increasing fibrillar

surface area and the potential for crosslinks between fibrils and interaction with other matrix components. Biomechanical properties of tendon may also change with no change in fibril diameters, as for example following scar formation (Frank *et al.*, 1992), where a change in the orientation of fibrils is likely to be responsible.

Much attention has recently been focused on non-enzymatic glycosylation and effect on mechanical properties of collagen. This is due mainly to the rôle of glycosylation products in the pathogenesis of the disease, diabetes. Andreassen and colleagues found that, whilst glycosylation per se does not necessarily influence mechanical properties, transformation into fluorescent compounds by the Maillard reaction results in an increase in the maximum stress withstood by the tissue (Andreassen & Oxlund, 1985). Furthermore, fluorescence intensity correlates with the breaking time of collagen fibres (Rolandi *et al.*, 1991). It is likely that other non-fluorescent, advanced glycosylation end-products also contribute to this increase in tendon fibre breaking time (Richard *et al.*, 1991).

Some evidence exists which, at first glance, suggest that non-collagenous proteins have an important effect on the mechanical properties of tendon (Partington & Wood, 1963). Hyaluronidase digestion resulted in a considerable weakening of the tendon. However, this effect may have been due to cleavage of the telopeptide region of the collagen molecule by proteinases contaminating the hyaluronidase preparation.

The crimped nature of collagen fibres determines the characteristics of the "toe" region of the stress-strain curve for tendon. The presence of crimp reduces the stiffness of the tendon during initial loading and the most likely function of crimp is to decelerate rapid loading thus preventing damage (Gathercole & Keller, 1991). Rat tail tendons have a pronounced "toe" region and operate within this range of extensions. In contrast, the major load bearing tendons in large mammals have a less pronounced "toe" region and operate within the linear region of the stress-strain curve. This may be because these tendons act as elastic energy stores and the straightening and refolding of crimp would not make a major contribution to this function (Ker, 1981).

1.12 Structure Function Relationships

Variation in matrix composition can not always be directly correlated with differences in mechanical properties. Regions of tendon subjected to different mechanical environments however, show adaptations, which presumably enable them to resist deformation. Digital flexor tendons can be divided into two regions; a tensional region and a compressional region, where the tendon passes over a joint. Several studies have addressed the differences in matrix composition between these two regions.

Water content is higher in the region subjected to compression in comparison to the tensional region. In bovine tendon values of 73% and 58% have been measured respectively (Vogel & Koob, 1989). Of the dry weight, collagen content in the tensional region equates to 82% while in the compressed region forms only 69%. In the latter region, the hydroxylysine and hydroxylysine/hydroxyproline ratios were higher suggesting an increased hydroxylation of collagen or a difference in collagen type (Okuda *et al.*, 1987a). Type I collagen however, was the predominant collagen in both regions.

Organisation of collagen also differs between the two regions. Merriles & Flint (1980) and Okuda and colleagues (1987b) found that collagen fibril diameters in the tensional region had a bimodal distribution while in the compressional region the distribution was unimodal with the peak diameter being smaller or equal to the thinnest fibrils in the tensional region.

Levels of the mature, trifunctional hydroxylysylpridinoline crosslink have been measured in bovine flexor tendon (Vogel & Koob, 1989) and give some of the highest recorded values for type I collagen matrices. This may relate to the high tensile forces experienced by this tendon *in vivo*. In the tensional region levels are 0·7 - 1·5 mol/mol collagen whilst in the pressure region the average is higher although in this region there is a difference between surface, middle and deep layers (1·05 ± 0·02, 1·17 ± 0·01, 1·31 ± 0·02).

Both the amount and type of GAG varies between the tension and pressure region of the tendon and has been studied in bovine flexor tendon by Vogel & Koob (1989). In the tensional region proteoglycan forms 0·2% of the dry weight, the predominant proteoglycan being the small dermatan sulphate core protein (PG II) with the associated dermatan sulphate GAG. In the pressure region proteoglycan accounted for 2% of the total dry weight. In this region the predominant proteoglycan had a large core protein and chondroitin sulphate GAG side chain.

Cell shape also differs between the two regions. In the tensional region cells are long and thin while in the pressure region they have a rounder appearance. The above variations demonstrate the ability of tendon to modulate its structural and material

properties in order to meet mechanical requirements distinct from the usual need for strength in tension.

1.13 Age Related Changes in Tendon

Maturation of tendons from the newborn animal until adulthood produces a series of well documented changes. Collagen concentration increases (Vailas *et al.*, 1985) and the number of cells per unit volume of tendon decreases in young growing rats (Inglemark, 1948a). Collagen fibril diameters are known to increase in both equine SDFT and human Achilles tendon changing from a unimodal distribution at birth to a bimodal distribution at maturity (Parry *et al.*, 1978a). Crosslinking between collagen molecules also changes. The number of reducible crosslinks (dehydro-HLNL and HLONL) declines until maturity (Cannon & Davison, 1977) at which point only negligible amounts remain (Robins *et al.*, 1973). This coincides with a rapid increase in the trifunctional crosslink, pyridinoline (Moriguchi & Fujimoto, 1978). Glycosaminoglycan content decreases (Vailas *et al.*, 1985), particularly hyaluronate and chondroitin sulphate (Scott & Hughes, 1986) and this decline coincides with the increase in fibril diameters.

Ageing of the extracellular matrix following the cessation of growth is much less well understood. One of the main reasons for this lack of understanding of the ageing process may be that many of the studies thus far have been carried out in short-lived species, such as rats and mice. In these species, following maturity, pyridinoline crosslinks continue to rise. However, in human Achilles tendon beyond the age of about 30 yr levels of pyridinoline begin to decrease (Moriguchi & Fujimoto, 1978). Similarly, in monkey skin samples pyridinoline was found to decrease in later life (Reiser *et al.*, 1987). Collagen fibril diameters decrease in the SDFT and suspensory ligament of the horse from maturity until senescence (Parry *et al.*, 1978a), while the ratio of type III collagen over type I + III collagen shows a progressive increase in several connective tissues with chronological ageing (Kern *et al.*, 1986). Ageing is also associated with non-enzymatic glycosylation of collagen and a progressive 'browning' of tissue (Kohn *et al.*, 1984; Sell & Monnier, 1989) which may explain the decreased susceptibility to collagenase digestion of tendon on ageing (Hamlin & Kohn, 1972).

The effect of ageing on mechanical properties of tendon is not clear. Hubbard and Soutas-Little (1984) could find no difference in tendon modulus in human tendons ranging in age from 16 - 88 yr and concluded that age effects, if any, could be masked by other factors such as health, diet, disease and exercise.

1.14 Effect of Physical Activity on Tendon

One of the first studies to assess the effects of training on tendons was carried out by Inglemark (1948b). The Achilles tendon of rabbits showed an increased wet weight, cross sectional area and collagen fibril diameter following 40 weeks of training. Mechanical tests were not, however, performed on these tendons. In a similar study on rabbits, training increased the tensile strength of various isolated tendons but this was not accompanied by an increase in tendon weight (Viidik, 1967). The maximal load the muscle-Achilles tendon-bone preparation could support prior to rupture was however unchanged (Viidik, 1967). The maximal tensile force needed to rupture the Achilles tendon in wild rats is higher than in domestic rats (Barfred, 1971) suggesting that physical activity increases the strength of this tendon; a finding that is supported by Vilarta & Vidal (1989). Kiiskinen & Heikkinen (1973) also reported an increase in tensile strength of patella tendons in growing mice following a 3 week training programme. In swine following 12 months of training, tensile strength of the extensor tendon was increased (Woo, 1980) and this was accompanied by hypertrophy and increased collagen concentration. In the flexor tendon however, following the same training programme no changes were observed (Woo, 1981). The lack of hypertrophy of the flexor tendon in response to exercise may be due to its inherent high strength (Woo, 1981). In young roosters following eight weeks of training collagen deposition was increased and the collagen contained fewer pyridinoline crosslinks suggesting greater matrix collagen turnover (Curwin *et al.*, 1988). Booth & Tipton (1972) could find no effect on collagen synthesis in rat Achilles tendon following one 30 min bout of exercise whilst collagen degradation was found to occur at a faster rate in the Achilles tendon of trained mature mice (Heikkinen & Vuori, 1972).

Adverse effects on tendon mechanical properties following training have also been reported. The ultimate tensile strength of rat Achilles tendon was found to decrease following 12-16 weeks of training (Sommer, 1987). Similar observations were made in goat flexor tendons following 3 and 6 months of training (Wilson, 1991). In young mice following one week of physical loading, an increase in tendon cross sectional area, fibril number and mean fibril diameter occurred (Michna, 1984). After 3 weeks of training however, all parameters fell below the control levels and were accompanied by signs of splitting of individual collagen fibrils.

Immobilisation also results in a loss of tensile strength in tendons (Woo, 1982). Following immobilisation of the tibialis anterior tendon in rabbit, ultimate tensile strength decreased but no change in hydroxyproline levels were observed (Loitz *et al.*, 1989). In rat patellar tendon immobilisation resulted in a decrease in both collagen and proteoglycan concentration (Vailas *et al.*, 1988). Reducible collagen crosslinks in periarticular tissue, including tendon, increased following immobilisation and GAG

content decreased (Akeson, 1961; Akeson & La Violette, 1964; Akeson *et al.*, 1967, 1973).

1.15 Tendon Injuries

The incidence of partial tendon rupture in horses is high. About 30% of horses in National Hunt training and many of those in other competitive events suffer from a tendon injury in one season. In human runners, 7% of all overuse injuries are to the Achilles tendon (Smart *et al.*, 1980), while in the horse the tendon most commonly affected is the SDFT (Webbon, 1973) particularly in the mid-metacarpal region (Smith, 1894). Several factors are recognised as predisposing to injury in both human and equine athletes. These are fatigue, lack of fitness, poor conformation, systemic disease and inco-ordinate action (Webbon, 1973).

1.16 Degenerative Changes

If stress is applied across a healthy muscle-tendon-bone system the tendon is seldom the point of rupture (McMaster, 1933; Stucke, 1950; Davidsson, 1954). This observation led Forsell (1952) to suggested, that a degenerative change in the tendon precedes rupture. Similarly, in human Achilles tendon, rupture is thought to follow a degenerative change (Arner *et al.*, 1959). In a study by Kannus *et al.* (1991) 891 spontaneously ruptured tendons were investigated and in every tendon there were obvious pre-existing histopathological alterations. They concluded that spontaneous rupture of a tendon was almost without exception, preceded by degenerative changes. Post mortem examination of equine flexor tendons also showed abnormal changes to the macroscopic appearance in transverse section of 30% of SDFTs and 3% of DDFTs (Webbon, 1977). These tendons, some from horses known to have been still actively racing, had a pink or purple core; the shape and size of the tendon however remained unchanged. Several histological studies have been carried out on human tendon tissue just post rupture and compared with apparently normal tissue (Józsa *et al.*, 1982, 1984 &1989). Following rupture no signs of inflammation or lipomatosis were present; the tenocytes however showed fine structurally marked hypoxic alterations (Józsa *et al.*, 1982). These included changes to mitochondria, endoplasmic reticulum, the number of ribosomes, nuclei and nucleoli and the number of lysosomes present. Structural alterations of collagen fibres were also observed. The average size of collagen fibre diameters was decreased (Józsa *et al.*, 1989) and longitudinal splitting and disintegration of collagen fibres observed in degenerated tendons (Józsa *et al.*, 1984).

Similar degenerative changes have been observed in the ageing rotator cuff tendon (Brewer, 1979).

The process by which tenocytes and collagen fibres degenerate is poorly understood; several possible pathways have been suggested. Arner and colleagues (1959) suggested that degeneration of the Achilles tendon in man is due to ischaemia and Strömberg (1971) proposed a similar aetiology in horses. Consistent with this, the most common site for SDFT injury is between the carpal and fetlock sheath (Smith, 1894); the area that Strömberg (1971) found to be less well vascularised than the rest of the tendon.

Ischaemia may lead to cell damage and subsequent tissue degeneration in several different ways (fig. 1.23). Firstly, ischaemia may result in tissue hypoxia and compromise energy metabolism in tenocytes resulting in cell damage. Ischaemia followed by reperfusion results in the generation of highly reactive free radicals which are capable of causing severe damage to many cell organelles. During loading of the tendon blood flow is impaired and when exercise has ceased normal blood flow returns. This may result in the generation of free radicals and a second pathway for cell damage in tendons. Alternatively, substantial temperature rises have been demonstrated to occur during high speed locomotion in the equine SDFT; this is partly due to the poor blood supply (Wilson & Goodship, 1991). Hyperthermia may represent another possible pathway for exercise mediated tendon cell damage. Damage to tendon cells would result in an inability to metabolise matrix components and therefore subsequent matrix degeneration (Fackelman, 1973). In individuals subjected to intensive training the demand for repair following matrix microdamage may well be increased and the inability to maintain the extracellular matrix due to cell damage would be amplified.

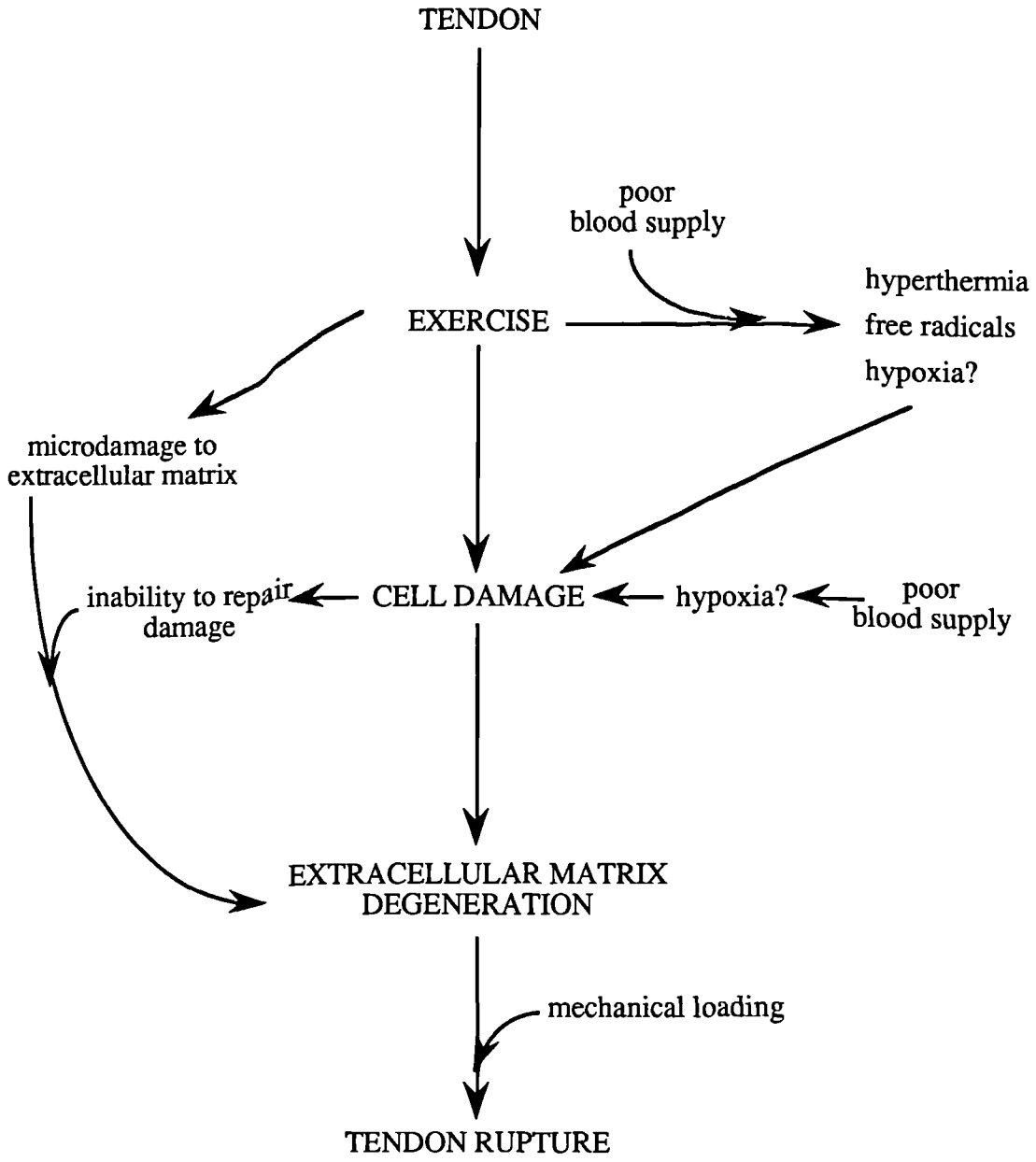


Fig. 1.23 Possible pathways for tendon degeneration.

1.17 Aim and Objectives

This thesis sets out to test a series of hypothesis developed under the overall aim of determining the cellular basis of tendon degeneration. The main questions to be resolved are:

1. Does the SDFT undergo progressive changes within the extracellular matrix during ageing and/or training which may result in alteration to material properties of the tendon? Do any such changes also occur in a tendon which rarely ruptures?

2. Are the macroscopic changes observed in degenerated SDF tendons accompanied by a change in the extracellular matrix composition?

3. Are mature equine tenocytes completely dependant on anaerobic metabolism or are they capable of aerobic metabolism and therefore require oxygen for maintenance of normal cellular function?

4. How do hypoxia, the generation of free radicals and hyperthermia affect the metabolism of equine tendon fibroblasts?

Chapter Two

EXTRACELLULAR MATRIX COMPOSITION OF EQUINE DIGITAL FLEXOR TENDONS

2.1 Introduction

A comparison of extracellular matrix composition of SDFT and DDFT

The superficial digital flexor tendon (SDFT) and deep digital flexor tendon (DDFT) are both tendons that flex the digit in the equine forelimb. They have however adapted to serve different mechanical rôles in equine locomotion. The SDFT is loaded early in the stride and acts as an elastic energy store experiencing high stresses and strains during high speed locomotion (Wilson, 1991). The DDFT on the other hand, is loaded later in the stride transmitting force generated by the muscle belly into movement of the metacarpo-phalangeal joint and is subjected to lower stresses and strains (Wilson, 1991). The mechanical properties of tendon can be attributed to the extracellular matrix. Such differences in mechanical function therefore might be expected to be reflected by differences in matrix composition.

Structure-function relationships have long been recognised in many connective tissues with diverse biological rôles. For example, the precise orientation of collagen fibrils in the cornea renders this tissue transparent and enables light to enter the eye (Maurice, 1984). The multidirectional orientation of helical fibrils within the tendon sheath enables it to resist force in all directions distinct from the usual parallel arrangement of straight fibrils able to resist axial load in tendon (Raspanti *et al.*, 1990). Adaptations to a more cartilage-like structure in regions of tendon subjected to compression enables this region to recover following deformation (Vogel & Koob, 1989). Furthermore, variations in tendons subjected to tensional loading exist between species, for example rabbit Achilles tendon contains 0·33 moles of the intermolecular crosslink hydroxylysylpyridinoline per mole of collagen while the respective range for bovine flexor tendon is 0·7 - 1·5 (Vogel & Koob, 1989). Differences can also occur between tendons subjected to tensional loading within the same species. Equine common digital extensor tendon has a unimodal distribution of fibril diameters while SDFT has a bimodal distribution (Parry *et al.*, 1978a) yet both tendons are loaded in axial tension, the former to a lesser extent than the latter.

Much of the available literature on tendon composition has been conducted on rat tail tendons due to availability and ease of sample collection (Betsch & Baer, 1980). Regarding the known variability between tendons, it is unlikely that these data are accurate for equine SDFT. Of the few studies carried out on tendons subjected to high stresses and strains and from long-lived species such as the horse, most have been

microscopic and histological studies (see chapter 1). It therefore seemed necessary to determine in a quantitative manner the biochemical composition of "normal" SDFT. Comparison with the DDFT may demonstrate adaptation for mechanical rôles *in vivo*.

Degenerative change and subsequent partial rupture is far more common in the SDFT than the DDFT (Webbon, 1977). This injury usually occurs in the central core of the mid-metacarpal region of the tendon. Separate analysis therefore, of central and peripheral tissue will enable any predisposition for injury in the central core to be evaluated. Comparison with the DDFT would determine whether any central/peripheral difference is peculiar to the SDFT.

Age and exercise related changes to the extracellular matrix of SDFT and DDFT

Ageing and training are both factors which may result in progressive changes in the nature of the extracellular matrix of tendon. Changes within tendon from maturity to senescence are not well documented and whether ageing alone is sufficient to produce degenerative changes remains unclear. Similarly, the effects of training remain unclear, some investigators have found an increase in the ultimate tensile strength of tendon following training (Vilarta & Vidal, 1989), while others report a decrease (Sommer, 1987; Wilson, 1991).

Comparison of extracellular matrix components in SDFT and DDFT from young and old groups of horses may demonstrate age related changes in both tendons. Differences in the young group between SDFT and DDFT would suggest an inherent difference probably due to their differing mechanical rôles *in vivo*. It is not possible to fully address this question without the examination of tendon tissue from foals immediately following birth. This was not feasible in this study so a young group of un-exercised horses were examined and compared with groups of un-exercised and exercised old horses. If differences between the two tendons only exist in the old group this would suggest an adaptation to mechanical loading or a tendon specific difference in the ageing process. Age related changes in only the SDFT might be attributed to the higher stresses and strains experienced by this tendon (Wilson, 1991) and therefore such changes should be most pronounced in a trained group of horses. These changes may be an adaptive response to enable the tendon to resist excessive deformation or may represent a degenerative change and reduction in material properties. Any changes occurring with either age or exercise which are more pronounced in the central zone of the tendon may indicate a degenerative change.

Hypothesis

The molecular composition and organisation of extracellular matrix in equine SDFT is related to the specialised mechanical rôle of the tendon. Ageing and associated training results in changes within the central zone of the SDFT which may alter the mechanical properties of this part of the tendon.

Objectives

1. To assess, quantitatively, the macromolecular composition of extracellular matrix and cellularity in the SDFT and compare with a flexor tendon which rarely shows degenerative changes and subsequent injury (i.e. the DDFT).

2. To determine whether matrix composition and organisation differs between central and peripheral tissue in either the SDFT or DDFT.

3. To characterise age and exercise related changes in extracellular matrix composition and organisation in both the SDFT and DDFT.

Experimental design

The proportion of dry weight matter constituting collagen in each tendon will be measured to give an indication of tendon material properties. Organisation of collagen within the matrix is also important (see chapter 1) and this will be assessed by identifying and quantifying collagen crosslinks, measuring fibril diameters, and microscopic examination of frozen longitudinal sections. Collagen type and glycosaminoglycan content and type may also be responsible for organisation of matrix (see chapter 1) and these will be assessed for further evidence for differences in collagen aggregation. Other factors which may indicate a difference in collagen type (amino acid content) or influence the degree of thermal stability of collagen (proline hydroxylation) will be measured. Non-enzymatic glycosylation of collagen, which results in a change in mechanical properties of the tendon, will be quantified by measuring tissue fluorescence and further analysed by separation of fluorescent crosslinks on a high-performance liquid chromatography (HPLC) column. Cellularity, which indicates capacity for matrix synthesis, will be measured by deoxyribonucleic acid (DNA) assay.

2.2 Methods

Tissue collection

Superficial digital flexor and deep digital flexor tendons were collected at a local abattoir from the forelimbs of Thoroughbred horses slaughtered for reasons other than tendon injury. A brief history and age of the animal was obtained from the owner and age verified by dental examination carried out by an experienced veterinary surgeon. A pre-mortem clinical assessment was carried out by an experienced veterinary surgeon to ensure that none of the selected horses had a clinically detectable tendon injury. Tendons were palpated in the standing horse and again on removal from the animal and any showing signs of thickening were excluded from the study. Horses were placed into one of three groups according to age and training history (see appendix 2). The first group (n = 5) consisted of young horses (age range 3 - 6 yr, mean age 4 yr) that had carried out only light work (group 1). The second and third groups were older horses (age range 8 - 16 yr) split into un-exercised (n = 6, mean age 14·8 yr) and exercised horses (n = 6, mean age 13·8 yr) respectively. Un-exercised horses (group 2) had been used for breeding or light riding purposes while the exercised animals (group 3) had been raced or evented.

The flexor tendons from either the left or right forelimb were selected randomly. A sample of tissue (2 cm length) was taken from the mid-metacarpal region of both the SDFT and DDFT (fig. 2.1) and stored at -20°C. Prior to analysis tendon samples were thawed at room temperature and separated into central and peripheral tissue (fig. 2.2). The central zone tissue is that which is susceptible to central core degeneration. Biochemical analyses were performed on tissue from both zones. A further length of tendon (1 cm) was snap frozen in an isopentane bath in dry ice at the time of collection (fig. 2.1) and stored at -70°C prior to preparation for electron microscopic analysis of both central and peripheral zone tissue.

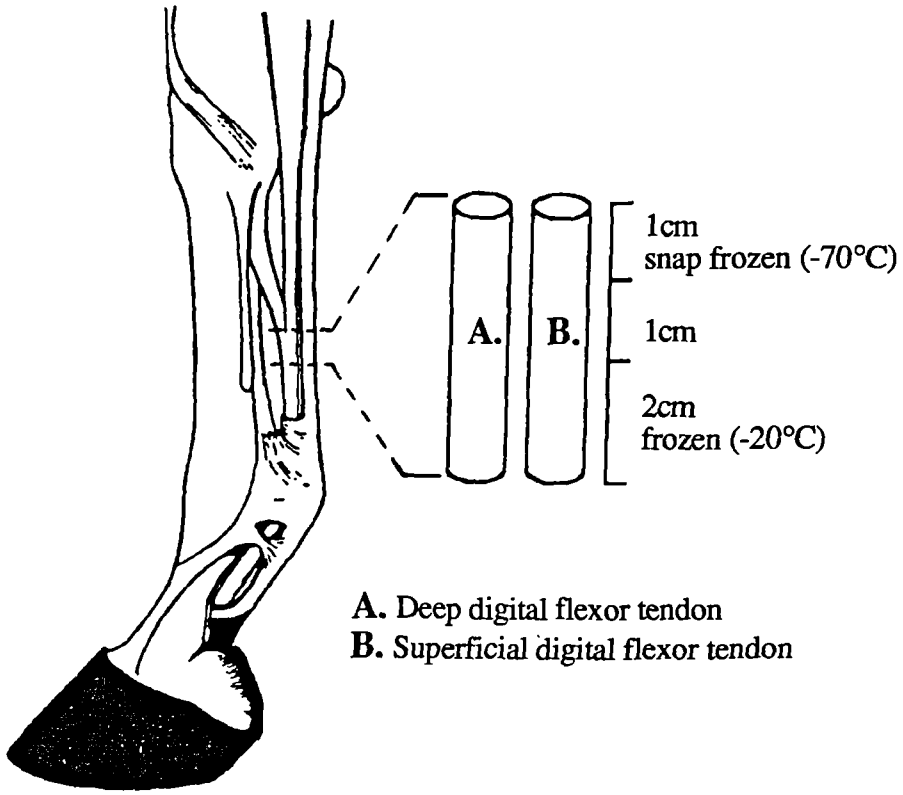


Fig. 2.1 Region of SDFT and DDFT taken for analysis.

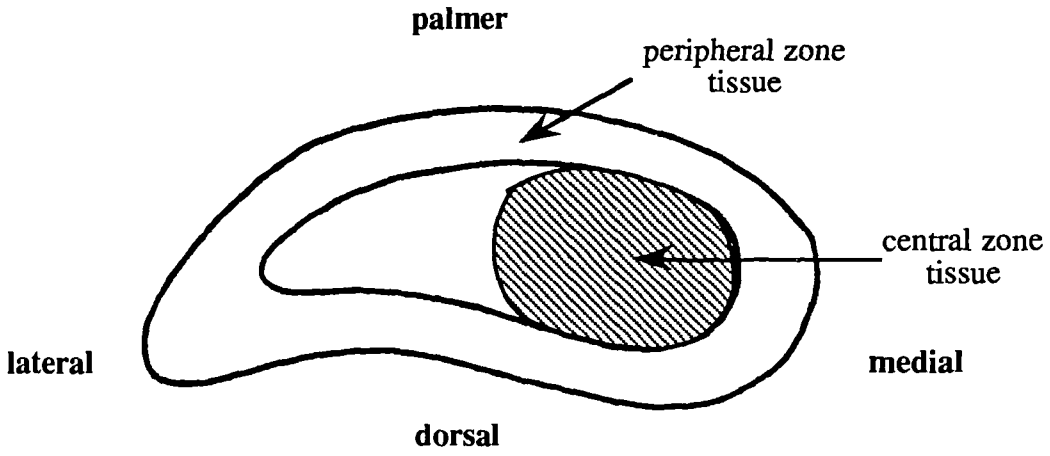


Fig. 2.2 Diagramatic representation of a transverse section through equine SDFT at the mid-metacarpal level showing central and peripheral zone tissue.

Water content

Approximately 100 mg of wet tissue were weighed out accurately from central and peripheral zones of each tendon sample on a digital balance (4 decimal places, Sartorius). Tissue was frozen at -70°C and freeze dried under vacuum (Christ Alpha I-5) until a constant weight was reached. Lyophilised tissue was then re-weighed to give a dry weight. Water content was expressed as a percentage of the wet weight.

Papain digestion

Prior to DNA assay and glycosaminoglycan (GAG) analysis tissue was solubilised by papain digestion. This process removes DNA and GAG associated proteins that interfere with the assays. Aliquots were also taken from the papain digest for hydroxyproline assay so that a direct comparison could be made between cellularity, collagen content and glycosaminoglycan content.

Following water content measurements, lyophilised tissue (10 mg) was suspended in 2 ml sterile PBS (phosphate buffered saline), pH 6·0, plus 5 mM cystiene.HCl and 5 mM EDTA (ethylenediamine tetra-acetic acid). Papain was added (125 μg/ml) and digestion carried out at 60°C for 24 hr. After this time tissue was completely solubilised.

DNA assay

Tissue cellularity was determined by assay of DNA. The method used is essentially that of Kim *et al.* (1988) utilising the bisbenzimidazole dye Hoechst 33258.

Hoechst dye (1 mg/ml in de-ionised water) was diluted immediately before use to 0·1 μg/ml in 10 mM Tris·HCl, pH 7·4, 1 mM EDTA and 0·1 mM NaCl. An aliquot of papain digest was mixed with dye solution to give a concentration of about 0·25 μg DNA/ml. Fluorescence was measured in a fluorometer (Perkin-Elmer 3000) at an excitation wavelength of 348 nm (slit width 5 nm) and emission wavelength 457 nm (slit width 10 nm). Fluorescence in the papain digests was also measured in the absence of dye and readings for DNA content corrected to account for background collagen-linked fluorescence (see below). DNA concentrations in the papain digests were calculated by comparison with a standard curve prepared with calf thymus DNA (1-10 μg DNA/ml sterile PBS) and diluted in dye solution to give a range of concentrations from 0·05 to 0·5 μg/ml. DNA content in tendon samples is expressed as μg DNA/mg dry wt tissue.

Glycosaminoglycan assay

Total sulphated glycosaminoglycan content was quantified by the method of Farndale *et al.*, (1986) using dimethylmethylene blue dye. Dimethylmethylene blue, a metachromatic dye, binds to sulphated GAGs producing a complex which absorbs light at 525 nm.

A working dye solution was made by dissolving dimethylmethylene blue at a concentration of 16 µg/ml in 40 mM glycine/HCl, pH 3·0 plus 40 mM NaCl. 100 µl of papain digest (containing approximately 5 µg GAG) were mixed with 3 ml dye solution in a plastic cuvette and the absorbence measured immediately at 525 nm in a spectrophotometer (Shimadzu UV-160). Concentrations were calculated by comparison with a standard curve prepared with purified bovine trachea chondroitin sulphate (0 - 10 µg in 3 ml dye). Results were expressed as µg chondroitin sulphate equivalent sulphated GAG/mg dry wt tissue.

Quantification of individual sulphated glycosaminoglycans

In a further six sets of superficial and deep flexor tendons individual sulphated GAGs were quantified by selective enzymatic degradation (Farndale *et al.*, 1986). Tendons were collected from horses (see appendix 2) (age range 9 - 20 yr, mean age 15·3 yr), with no previous clinically detectable tendon injury and samples taken as described above. Horses were not separated into exercised and un-exercised groups nor was tissue separated into central and peripheral zones for this part of the work, as no difference between either GAG content or fibril diameter had been observed between the different zones or groups in the previous study.

Tendon tissue was papain digested by the method described above and three 250 µl aliquots (approx. 100 µg GAG/ml) were taken from each papain digested sample. To the first, 250 µl of chondroitinase ABC (0·1 U/ml 50 mM Tris, pH 8·0) was added; to the second 250 µl chondroitinase AC (0·1 U/ml 50 mM Tris, pH 8·0) and to the third 250 µl of 50 mM Tris, pH 8·0. Digestion was carried out at 37°C for 30 min.

Following digestion, aliquots of each sample were mixed with dimethylmethylene blue dye and absorbence measured at 525 nm. Chondroitin sulphate (CS) concentrations were calculated by subtraction of the absorbence following chondroitinase AC digestion (digestion of CS only) from the total (reading after incubation with Tris buffer only) and comparison with a CS standard curve (0 - 10 µg in 3 ml dye). Dermatan sulphate (DS) was calculated by subtraction of the reading following chondroitinase ABC digestion (digestion of CS and DS) from the reading following chondroitin AC digestion and comparison to a DS standard curve (0 - 10 µg

in 3 ml dye). The reading following chondroitinase ABC digestion represents keratan sulphate (KS). Digestion of GAG standards was also carried out with each of the enzymes. Individual GAGs are expressed as a percentage of the total.

Collagen fibril diameters

Snap frozen samples of tissue (see fig. 2.1) were thawed and fixed in a 2·5% glutaraldehyde and 2% paraformaldehyde solution. Washing was then carried out in 0·1 M sodium cacodylate buffer (pH 7·2) and samples fixed in 1% osmium tetroxide. Following dehydration in increasing concentrations of ethanol (10% - absolute), blocks were cut from the central and peripheral zones of the tendon. Blocks were mounted in resin (Taab 812) and thin (90 nm), transverse sections cut on a Reichert Jung Ultracut E. microtome. Sections were double stained with Reynold's lead citrate and uranyl acetate and examined on a Phillips (model 3000) electron microscope. Random areas of each section were photographed at 30 000 and 100 000 times magnification. Fibril diameters were measured on the 100 000 times photomicrograph using a Numonics 2206 digitiser.

Collagen content

The imino acid hydroxyproline is virtually restricted to collagen and is present at a level of 14% and therefore an assay of hydroxyproline can be used to quantify collagen content. The method used for the assay of hydroxyproline was developed from that of Bergman & Loxley (1963) and Bannister & Burns (1970).

100 µl were taken from the papain digest and hydrolysed in 6 M HCl (0·5 mg dry wt/ml) at 110°C for 24 hr. The hydrolysate was then dried under a vacuum and the residue dissolved in de-ionised water (approx. 5 µg hydroxyproline/ml). Hydroxyproline was assayed on an automated continuous flow analyser (Chemlab Instruments Ltd). The reaction involves the oxidation of hydroxyproline by chloroamine T followed by coupling with dimethylaminobenzaldehyde. The resulting coloured product is measured at 550 nm. Hydroxyproline concentrations were calculated by comparison with a standard curve (1 - 10 µg hydroxyproline/ml) and collagen content calculated assuming hydroxyproline to be present at 14%. Collagen content was expressed as a percentage of the dry weight.

Amino acid analysis

Amino acid analysis was carried out on SDFT and DDFT central tissue from three horses in both the young group and old group. Exercised and non-exercised groups were not separated for this analysis.

Lyophilised tissue was hydrolysed in 6 M HCl (5 mg/ml) for 24 hr at 110°C. The hydrolysate was then rotatory evaporated to dryness and the residue taken up in de-ionised water. A sample containing approximately 2·6 mg collagen was dried and re-dissolved in 4 ml 0·2 M sodium citrate, pH 2·2 (LKB loading buffer).

Amino acids were assayed by applying to an LKB 4400 amino acid analyser which basically consists of a cation exchange column and detection system. Samples were filtered through a 0·2 µm filter and 40 µl applied to the column at a pH 2·2. At a low pH all the amino acids bind to the column and as the pH of eluting buffers is gradually increased to 8·6 and the ionic strength of the buffer increased to 0·5 M, amino acids are eluted from the column. Amino acids are detected with ninhydrin and identified and quantified by comparison with a standard mixture of amino acids.

Collagen type

Lyophilised tissue samples (approx. 5 mg) were suspended in 0·5 ml 70% formic acid and 10 mg CNBr (cyanogen bromide) were added to each. CNBr digestion was carried out at 30°C for 5 hr and after this time solubilisation was complete. Digestion was stopped by a 1 in 10 dilution with de-ionised water and digests freeze-dried. Lyophilised samples were washed once with 1 ml of de-ionised water and re-dried. The resulting peptides following CNBr digestion were taken up in 0·5 ml sample buffer (125 mM Tris, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 0·01% bromophenol blue) and heated for 30 min at 60°C to ensure dissociation of the α chains. CNBr peptides were separated by SDS-PAGE (polyacrylamide gel electrophoresis)("Hoffer" design apparatus, LKB) on a 12·5% gel by the method of Laemmli (1970). Gels were stained with Coomassie brilliant blue (50 µg/ml) in destain solution (4:3:33, methanol : acetic acid : distilled water). Gels were then destained overnight in destain solution. Standards of purified type I and III collagen prepared from equine foetal skin by salt fractionation, and CNBr digested as described above, were also separated by electrophoresis (see fig. 2.3) along with the samples. The bands α1(I)CB8 and α1(III)CB5 were used to quantify type I and III collagen respectively. These peptide bands are used because neither peptide is involved in crosslinkage and so always migrate to the same positions on the gel. Gels were scanned on an enhanced

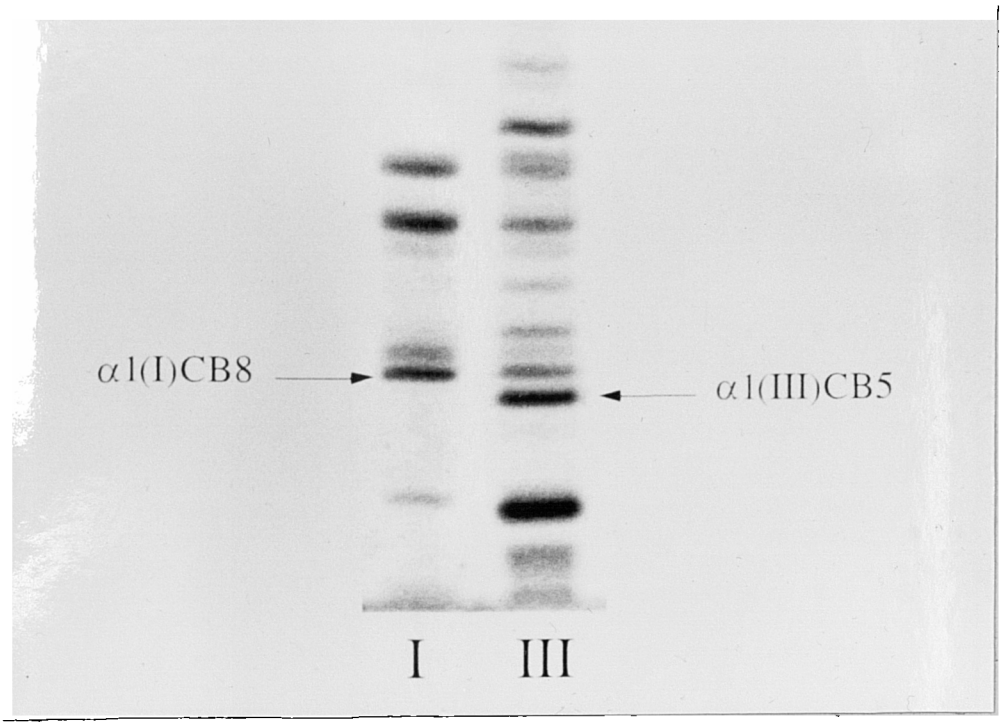


Fig. 2.3 Separation of CNBr peptides from purified type I and type III equine collagen standards on a SDS polyacrylamide gel by electrophoresis.

laser densitometer (LKB Ultroscan XL) and the type III collagen content calculated as a percentage of type I + type III collagen.

Immunohistochemical localisation of type III collagen

Frozen longitudinal sections were cut from both the SDFT and DDFT on a Bright cryostat / microtome (model OTF/AS/MR/LT). A 1 cm length of tendon was mounted in embedding medium (Tissue-Tek) on a sectioning block and trimmed until the widest part of the tendon was reached. 10 μm thick sections were then cut at -30°C and stuck to slides coated with a chrome alum-gelatin adhesive (Bancroft & Cook, 1984).

Antibodies to whole αIII chains were used to determine the distribution of type III collagen. Prior to incubation with the antibody sections were soaked in PBS (1 M NaH_2PO_4, pH 7·4, 1·2 M NaCl) overnight. The goat anti-type III collagen antibody was diluted 1 in 40 in PBS and 100 μl spread evenly over each section. Incubation with the first antibody was carried out for 2 hr at room temperature. Following incubation, 30 min. washes (x3) were carried out in PBS. The second rabbit anti-goat IgG (whole molecule) fluorescein isothiocyanate-labelled antibody was diluted 1 in 32 in PBS and 100 μl applied to each slide. Incubation was carried out at room temperature for 3·5 hr. Further 30 min. washes (x3) were carried out in PBS. Sections were protected by covering with a glass coverslip with a spot of glycerol/PBS solution (citifluor) underneath. Controls were conducted by parallel incubation of each section with a goat anti-type I antibody by the method described above and also by incubation of sections with goat serum instead of a first antibody. Antibody specificity was checked prior to use, on whole α chain standards of type I and III collagen prepared from equine foetal skin (see chapter 6).

Sections were examined under a fluorescent light microscope (Leitz Dialux 20) at a magnification of 10 or 25 times and photographs taken.

Crosslink analysis

The method used for the preparation and measurement of collagen crosslinks was that of Sims & Bailey (1992). Approximately 100 mg wet tissue was taken from the central zone of each tendon and suspended in 2·5 ml of 0·025 M PBS. Reduction of reducible crosslinks was carried out by the addition of 200 μl potassium borohydride (20 mg/ml in N, N - dimethylformamide). Incubation with the reducing agent was carried out at room temperature for one hour and the reaction then stopped by lowering the pH of the mixture to 3·0 by the addition of glacial acetic acid. Tissue was removed from the solution by centrifugation and washed twice with de-ionised water before

lyophilisation. Dried tissue was re-weighed and transferred to screw-top hydrolysis tubes. 6 M HCl (5 ml) was added and tissue hydrolysed for 24 hr at 110°C. Hydrolysates were dried under vacuum and the residue dissolved in 1 ml de-ionised water. A small aliquot (10 µl) was taken for a hydroxyproline assay which was carried out as described previously. The remainder of the hydrolysate was fractionated on a CF1-cellulose column by the following method.

1 ml of glacial acetic acid was added to the sample followed by 4 ml of butan-1-ol to give a butanol : acetic acid : water ratio of 4 : 1 : 1 referred to as the organic phase. CF1-cellulose was prepared by washing in de-ionised water (x4) and pouring into a sintered glass funnel where three bed volumes of "organic phase" were passed through under vacuum. The washed CF1-cellulose was then resuspended in organic phase at a concentration of 5% (w/v). A column of CF1-cellulose was prepared by removing the top of the bulb of a 3 ml disposable plastic Pasteur pipette and inserting a small plug of glass wool into the lower end. CF1 slurry was poured into the pipette until it reached a bed height of 8 cm and the bed was then settled by washing the column with 8 ml of organic phase.

Each hydrolysate sample dissolved in organic phase (6 ml) was applied to a separate CF1 cellulose column. Sample tubes were washed with 2 x 2 ml of organic phase and this also added to the column. Under these conditions the crosslinked amino acids remain adsorbed to the cellulose. Non-crosslinked amino acids were eluted from the column by washing with a further 16 ml of organic phase and the effluent was discarded. Collecting vessels were replaced with 20 ml tubes (Sterilin) and crosslinked amino acids eluted from the column with 8 ml of de-ionised water. The effluent was collected and centrifuged for 15 min. at 1000g. The lower aqueous phase, which contains the crosslinked amino acids, was removed with a long glass Pasteur pipette. This was freeze dried and the residue dissolved in 200 µl of 0·20 M sodium citrate buffer, pH 2·2 (LKB loading buffer). Samples were filtered (0·2 µm filter), and 40 µl applied to an LKB 4400 amino acid analyser configured for the separation of collagen crosslinks. Crosslinked compounds were detected with ninhydrin and identified by comparison with a standard crosslink preparation (fig. 2.4). Comparison with the amino acid, leucine, was used to quantify crosslinks and the leucine equivalent factor for each particular crosslink (previously determined) included in the calculation. Results were expressed as moles crosslink per mole collagen.

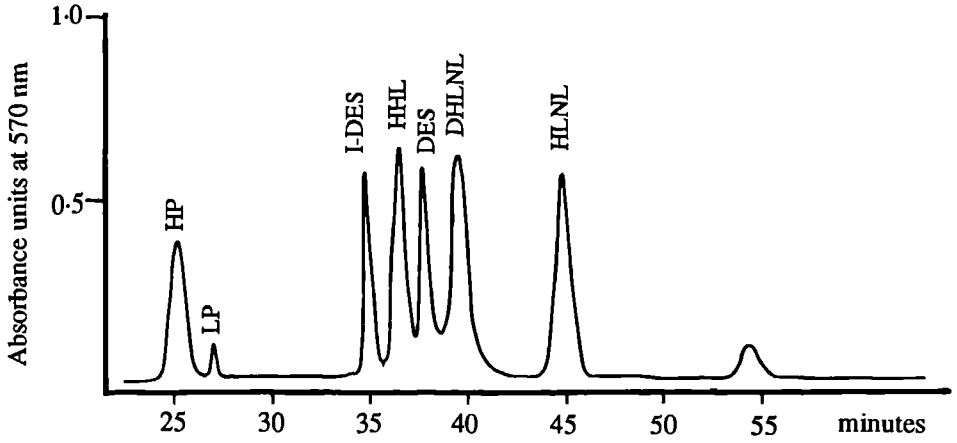


Fig. 2.4 Typical elution profile of cross-linking amino acids from collagen and elastin. HP (hyroxylysylpyridinoline), LP (lysylpyridinoline), I-DES (isodesmosine), HHL (histinohydroxylysinonorleucine, DES (desmosine), DHLNL (dihydroxylysinonorleucine), HLNL (hydroxylysinonorleucine).

Collagen-linked fluorescence

The extent of collagen glycosylation and subsequent conversion to advanced Maillard products (see chapter 1) was assessed by measuring the fluorescent nature of the tissue. These determinations were carried out as part of the DNA assay (background collagen-linked fluorescence). Samples of papain digested tissue were mixed with 10 mM Tris, pH 7·4 containing 1 mM EDTA and 0·1 mM NaCl (0·15 mg collagen/ml) and fluorescence measured in a fluorometer (Perkin-Elmer 3000) at an excitation wavelength of 348 nm (slit width 5 nm) and emission wavelength of 457 nm (slit width 10 nm). Fluorescent units are expressed per mg of collagen.

Glycosylated crosslinks

Assays for glycosylated crosslinks were carried out on SDFT and DDFT central tissue from three horses in both the young group and old group. Exercised and non-exercised groups were not separated for this analysis as no difference in collagen-linked fluorescence was observed between groups 2 & 3. 100 mg wet weight tissue was hydrolysed, as described above. The hydrolysate residue was dissolved in de-ionised water (approx. 1 mg collagen/ml) and 1 ml applied to a CF1 cellulose column. Columns were run in the same way as those for crosslink analysis on the amino acid analyser. Effluents were dried (aqueous and organic phases were not separated) and dissolved in 1 ml de-ionised water. 100 µl (containing equivalent to 100 µg collagen) were transferred to a HPLC ampoule, dried and dissolved in 100 µl 10% HFBA (heptafluorobutyric acid). Reversed-phase HPLC was run using a 12 - 34% gradient of

Fig. 2.5 HPLC elution profile of a standard preparation showing peaks believed to correspond to glycosylated crosslinks. P, pentosidine. Hydroxypyridinoline (HP) and its stereo isomer can also be seen on the trace.

acetonitrile in water containing 0·05 M HFBA over 44 min. Standard preparations containing glycosylated compounds were separated along with the samples (fig. 2.5).

Statistical analysis

Unless otherwise stated, statistical significance was evaluated using a mixed model ANOVA test where horse was a random effect and tendon (SDFT or DDFT), zone (central or peripheral) and group (young, old un-exercised or old exercised) were fixed effects. The level of significance was taken as $p \leq 0{\cdot}05$. Data are presented as mean ± S.E.M.

2.3 Results

The results from the analysis of SDFTs are summarised in table 2.1, table 2.2 shows a summary of results obtained from DDFTs.

Water content

Water content for corresponding zones was not significantly different between groups for either the SDFT or DDFT. The central region of the DDFT had a significantly lower water content than the peripheral zone of the same tendon ($p < 0{\cdot}001$) for all groups. No difference in water content was seen between central and peripheral tissue in the SDFT (fig. 2.6).

DNA content

No differences were observed between corresponding zones in groups 2 and 3 in either the SDFT or DDFT (i.e. no exercise effect) and therefore these two groups were combined to form one group of old horses for further statistical analysis. DNA content did not differ significantly between the central and peripheral zones of either the SDFT or DDFT. A comparison between the central zone of SDF and DDF tendons showed less DNA in the DDFT than the SDFT (fig. 2.7). This difference was not significant for the young group of horses but was highly significant for the old group of horses ($p < 0{\cdot}001$, $n = 12$). DNA content of central zone tissue did not differ

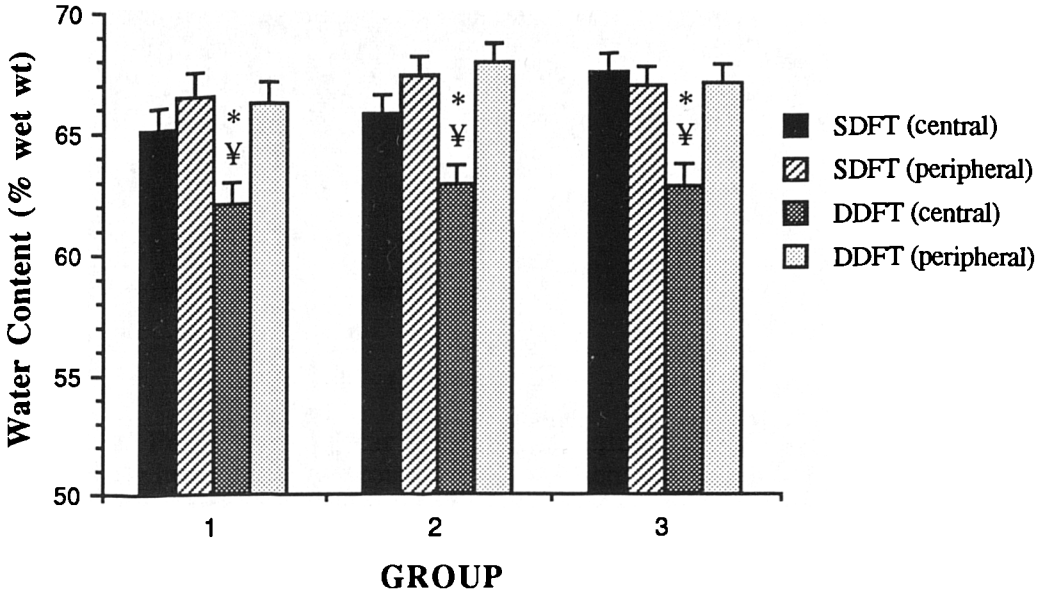


Fig. 2.6 Percentage water content in equine flexor tendons. Group 1 = young horses (n = 5), group 2 = old un-exercised horses (n = 6), group 3 = old exercised horses (n = 6). ¥ denotes a significant difference relative to the peripheral zone of the tendon and * a significant difference relative to the same zone in the SDFT.

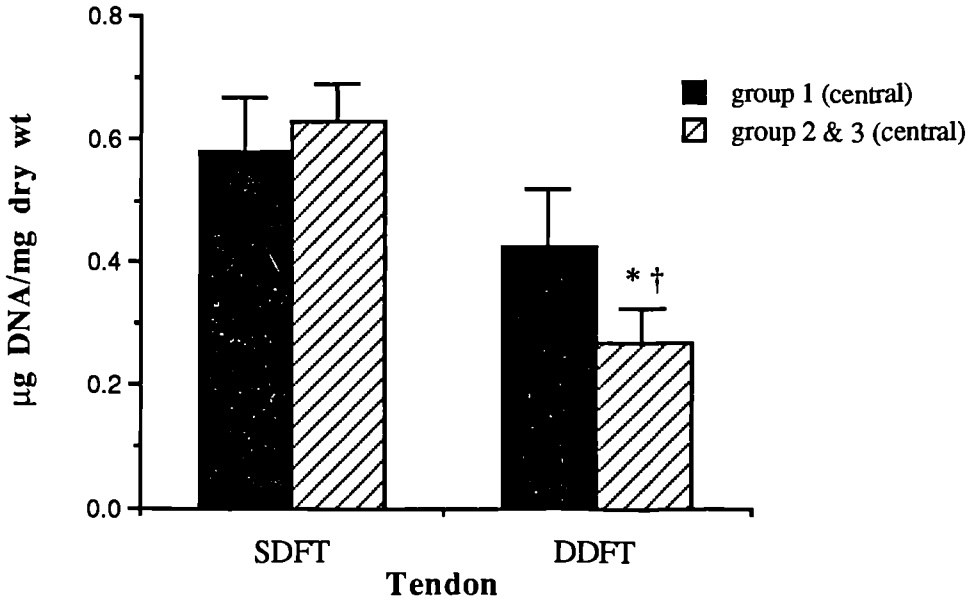


Fig 2.7 DNA content in the central zone of equine SDFT and DDFT. Group 1 = young horses (n = 5), group 2 & 3 = old horses (n = 12). † denotes a significant difference relative to group 1, and * a significant difference relative to the SDFT.

between the young and old groups of horses for the SDFT but in the DDFT there was a significant decrease in DNA content with ageing ($p < 0{\cdot}02$).

Total sulphated glycosaminoglycan content

No differences were observed between corresponding zones in groups 2 and 3 in either the SDFT or DDFT (i.e. no exercise effect) and therefore these two groups were combined to form one group of old horses for further statistical analysis. Total sulphated chondroitin sulphate equivalent glycosaminoglycan content did not differ between central and peripheral zones of either tendon. DDFTs had a higher GAG content than the SDFT (fig. 2.8) and this difference was significant in the old group of horses ($p < 0{\cdot}001$, $n = 12$). GAG content was lower in the old group of horses than the young group for both tendons, but these differences were not significant.

Proportion of individual sulphated glycosaminoglycans

A two-tailed paired t-test was used to test for significance between the two groups of data (SDFT & DDFT) obtained from the analysis of different glycosaminoglycans. The relative proportions of both dermatan sulphate and keratan sulphate differed significantly ($p < 0{\cdot}02$, $n = 6$) between the SDFT and DDFT (fig. 2.9) (%DS: SDFT $19{\cdot}2 \pm 0{\cdot}5$, DDFT $10{\cdot}0 \pm 2{\cdot}1$; %KS: SDFT $12{\cdot}2 \pm 1{\cdot}4$, DDFT $25{\cdot}0 \pm 2{\cdot}0$). Chondroitin sulphate accounted for $68{\cdot}5\% \pm 1{\cdot}8$ in the SDFT and $64{\cdot}8\% \pm 0{\cdot}9$ in the DDFT. These values were not significantly different.

Collagen fibril diameters

Fibril diameter data was analysed statistically using a general linear model to apply a Bonferroni (Dunn) t test to ranked values. No differences were observed between central and peripheral zones of the tendon or between groups. Combined data for the SDFT gave a fibril diameter median value of 47·8 nm (range 9·2 - 363·7 nm) while the median value for the DDFT was 122·8 nm (range 17·7 - 458·4 nm) and these values were significantly different ($p < 0{\cdot}01$, $n = 17$). The distribution of fibril diameters is represented graphically in figure 2.10. Fibrils with a diameter less than 150 nm represent 90% of the total in SDFTs and 55% of the total in the DDFT. Figures 2.11 & 2.12 show electron micrographs of the SDFT and DDFT respectively.

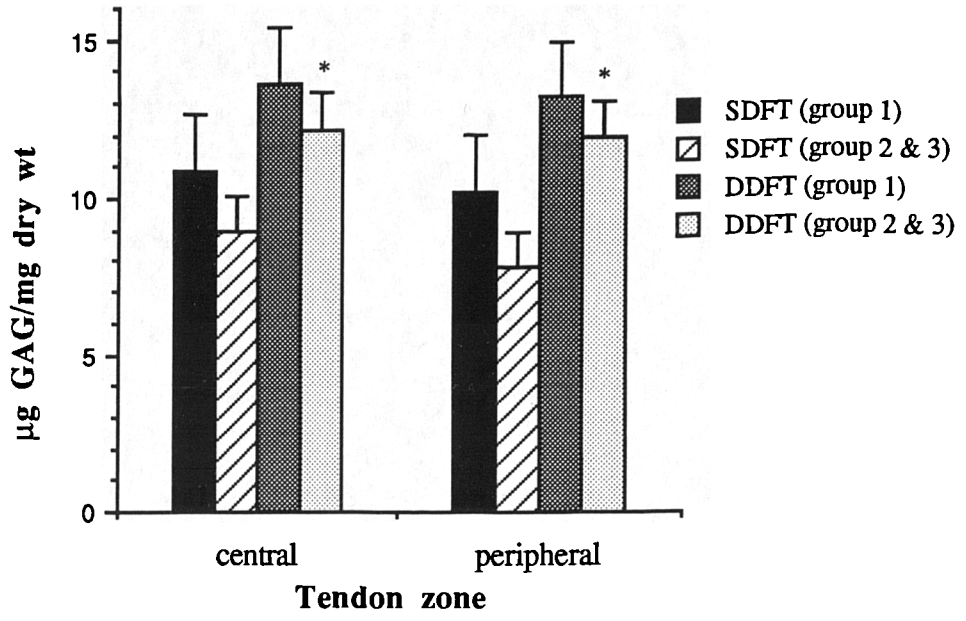


Fig. 2.8 Total sulphated chondroitin sulphate equivalent glycosaminoglycan content in equine flexor tendons. Group 1 = young horses (n = 5), group 2 & 3 = old horses (n = 12). * denotes a significant difference relative to the SDFT.

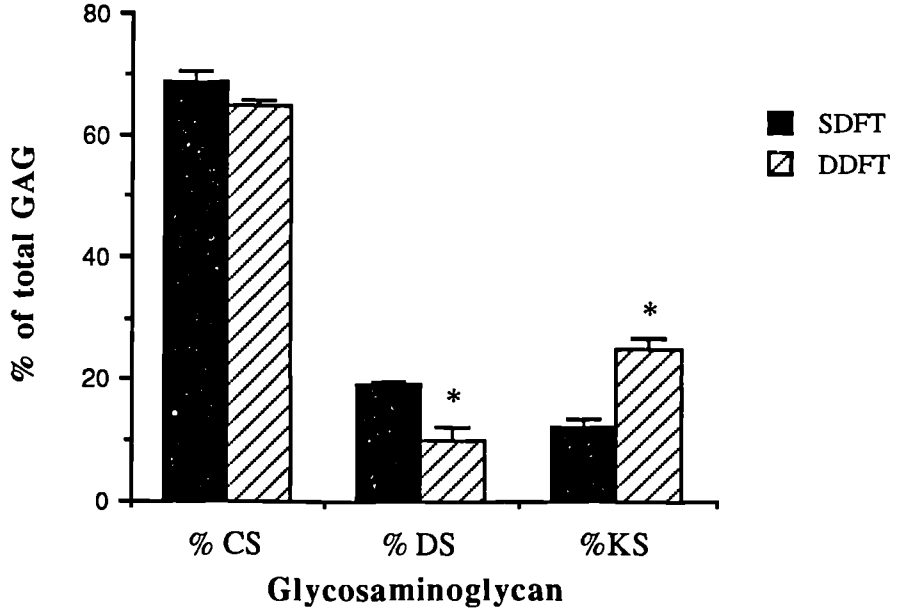


Fig. 2.9 Proportion of different glycosaminoglycans in equine SDFT and DDFT. (n = 6). * denotes a significant difference relative to the SDFT.

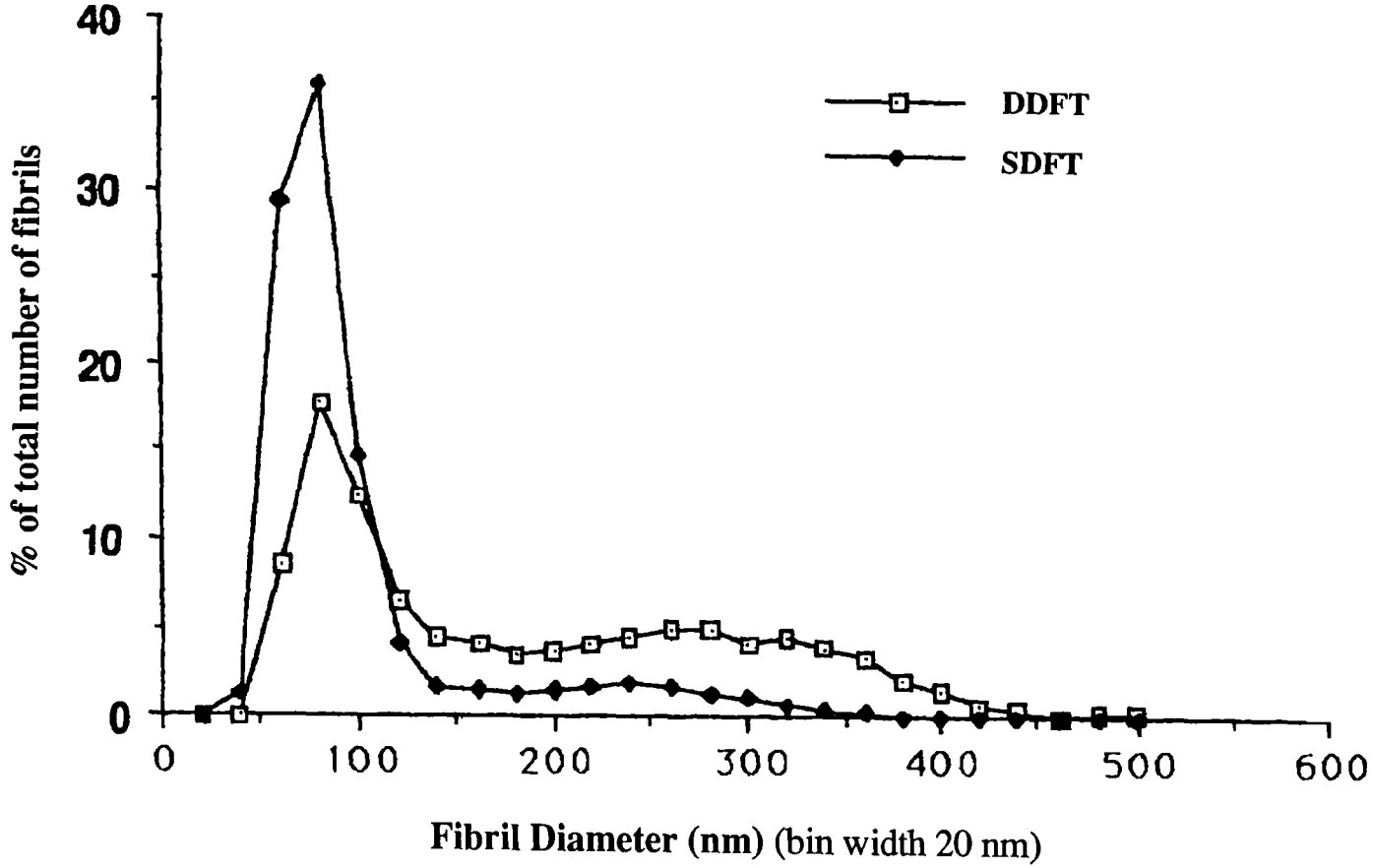


Fig. 2.10 Fibril diameter distribution in equine SDFT and DDFT.

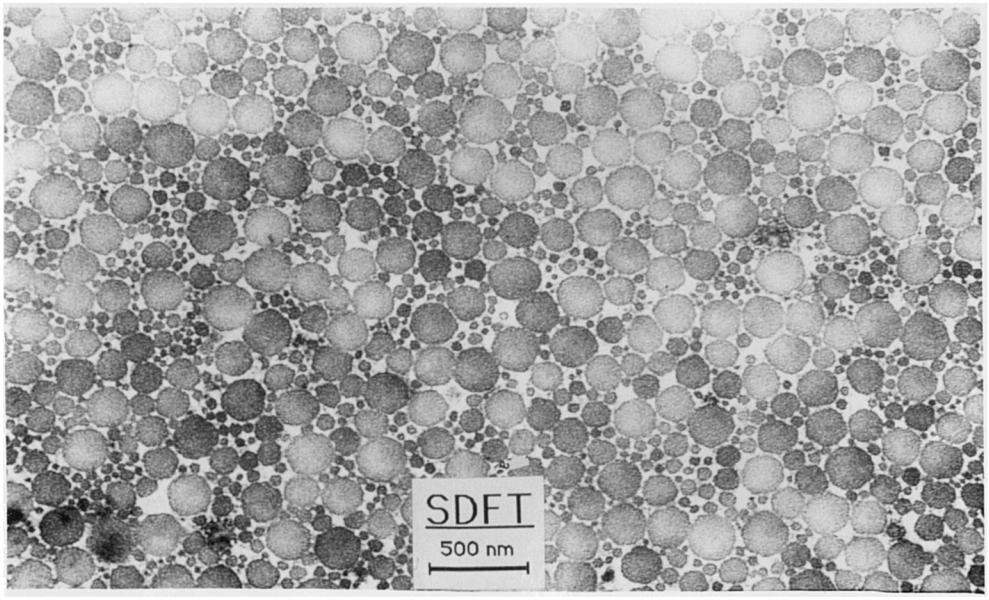


Fig. 2.11 Electron micrograph of a transverse section of equine SDFT.

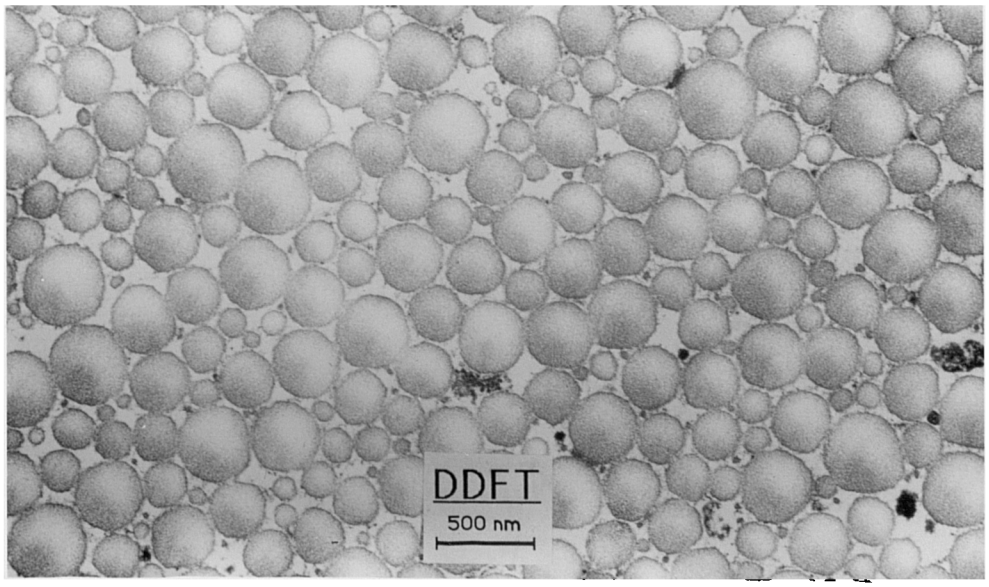


Fig. 2.12 Electron micrograph of a transverse section of equine DDFT.

Collagen content

Collagen formed about 75% of the dry weight of both the SDFT and DDFT. There were no differences in percentage collagen content between any of the groups for central or peripheral samples or between the SDFT and DDFT.

Amino acid analysis

Samples from different groups were pooled for amino acid analysis and comparisons made between the SDFT (n = 6) and DDFT (n = 6). Statistical significance was evaluated using a two-tailed non-paired t test. The hydroxylation of proline and lysine residues did not differ between tendons (% hydroxylation of proline: SDFT 37 ± 1, DDFT 38 ± 1; % hydroxylation of lysine: SDFT 22 ± 1, DDFT 22 ± 1). The number of serine, glutamate and isoleucine residues was significantly higher in the SDFT than in the DDFT (fig. 2.13 & 2.14).

Percentage type III collagen

Type III collagen content was determined by separation of CNBr peptides on a polyacrylamide gel and quantified by scanning the resulting peptide bands on a laser densitometer (fig. 2.15). No differences were observed between corresponding zones in groups 2 and 3 in either the SDFT or DDFT (i.e. no exercise effect) and therefore these two groups were combined to form one group of old horses for further statistical analysis. The SDFT had a higher proportion of type III collagen than the DDFT (fig. 2.16). This difference was significant for both central ($p < 0.001$, $n = 12$) and peripheral ($p < 0.01$, $n = 12$) zones in group 2 & 3 combined tendons and for the central zone of group 1 tendons ($p < 0.01$, $n = 5$). The highest value obtained for type III collagen was in the central zone of the SDFT in the group of old horses (15·7% ± 1·6) and this value was significantly higher ($p < 0.001$, $n = 12$) than the peripheral zone of the same tendons (8·0% ± 1·6).

Immunohistochemical localisation of type III collagen

Examination under the microscope of frozen sections labelled with fluorescein isothiocyanate tagged anti-type III collagen antibody showed a brightly stained region at the edges of fibre bundles demonstrating that type III collagen is located predominantly in the endotenon membrane around the subfascicular unit (fig. 2.17 a & b). The possibility that this was an edge effect due to incomplete diffusion of antibody into the fibre bundles was ruled out as sections stained with an antibody to type I collagen

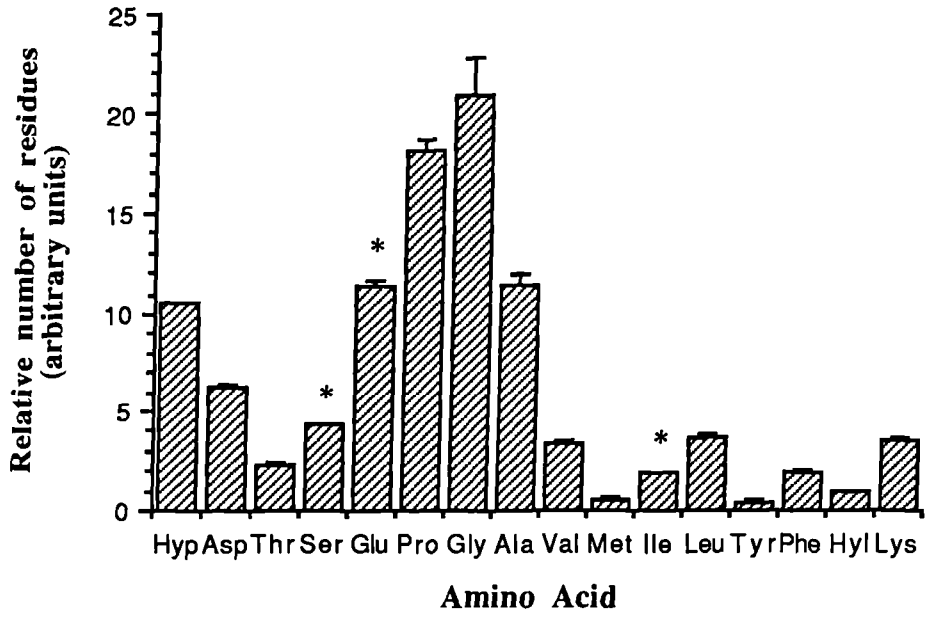


Fig. 2.13 Amino acid content of equine SDFT. (n = 6) * denotes a significant difference relative to the DDFT.

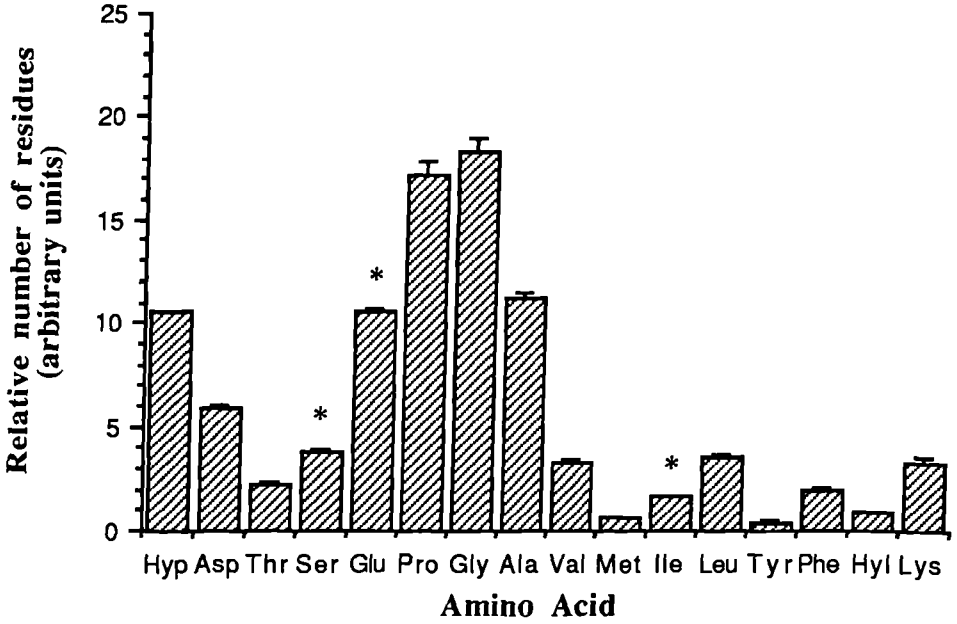


Fig. 2.14 Amino acid content of equine DDFT. (n = 6) * denotes a significant difference relative to the SDFT.

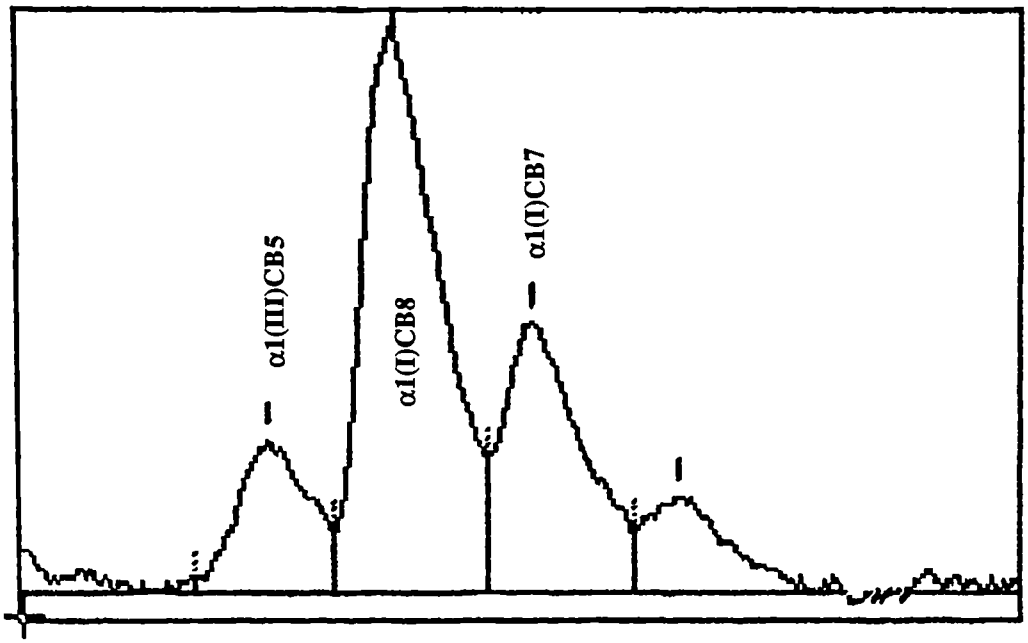


Fig. 2.15 Trace obtained from the laser densitometer scan of peptide bands following CNBr digestion and SDS-PAGE of central zone tissue from a group 2 & 3 SDFT.

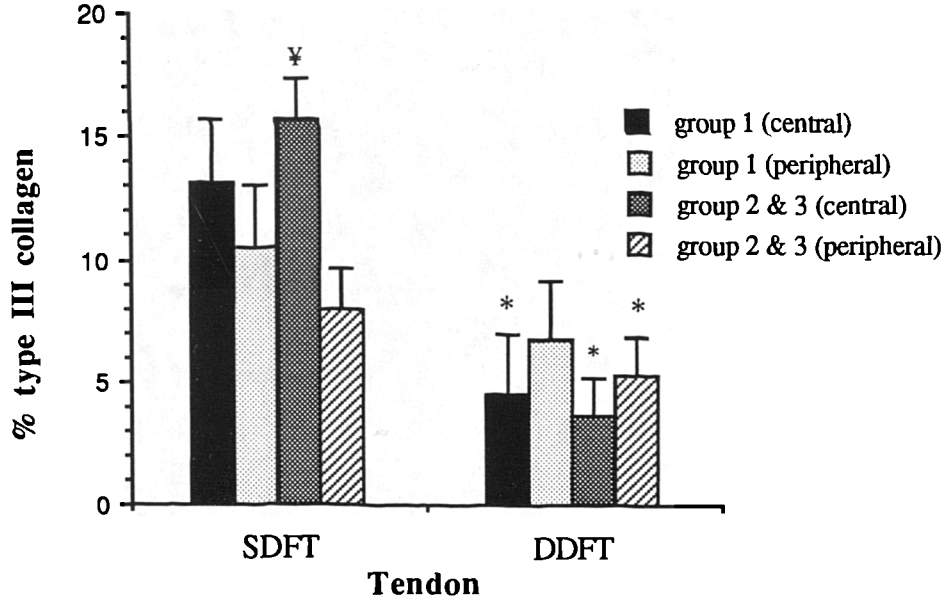


Fig. 2.16 Percentage type III collagen of type I + III collagen in equine flexor tendons. Group 1 = young horses (n = 5), group 2 & 3 = old horses (n = 12). * denotes a significant difference relative to the SDFT, ¥ a significant difference relative to the peripheral zone of the tendon.

showed an even fluorescence across the subfascicular unit (fig. 2.17 c). The higher levels of type III collagen in SDFTs compared to DDFTs detected by SDS-PAGE of CNBr digests may be due to a smaller diameter of fascicles in the SDFT compared to the DDFT. No non-specific binding of antibodies to either the goat serum or to the adhesive coating on the slide was observed.

Crosslink analysis

The mature crosslink hydroxylysylpyridinoline (HP) was the predominant crosslink in both SDFTs and DDFTs (fig. 2.18). Lysylpyridinoline (LP) and histidinohydroxylysinonorleucine (HHL) were also detected but only at very low levels (approx. 0·04 moles/mole collagen for both LP and HHL) and were therefore not further analysed. Only trace amounts of the reducible crosslinks were present in the tissue samples.

In all groups SDFTs had significantly ($p < 0·002$) higher levels of HP than DDFTs (fig. 2.19). HP did not differ significantly between groups in the SDFT. In the DDFTs however, group 3 tendons had significantly fewer HP crosslinks than group 2 tendons ($p < 0·002$, $n = 6$).

Collagen-linked fluorescence

Collagen-linked fluorescence did not differ significantly between central and peripheral samples or between SDFTs and DDFTs. No differences were observed between corresponding zones in groups 2 and 3 in either the SDFT or DDFT (i.e. no exercise effect) and therefore these two groups were combined to form one group of old horses ($n = 12$) for further statistical analysis. In both tendons group 2 & 3 combined had a significantly higher collagen-linked fluorescence than group 1 tendons ($p < 0·001$) and collagen-linked fluorescence correlated well ($R^2 = 0·91$) with the age of the horse (fig. 2.20).

Glycosylated crosslinks

Pentosidine, the only advanced glycosylation end-product characterised to date, was not detected in any of the samples. No other peaks that may be due to crosslinks formed by non-enzymatic glycosylation were observed in either the SDFT or DDFT following the preparation and analysis described above (fig. 2.21).

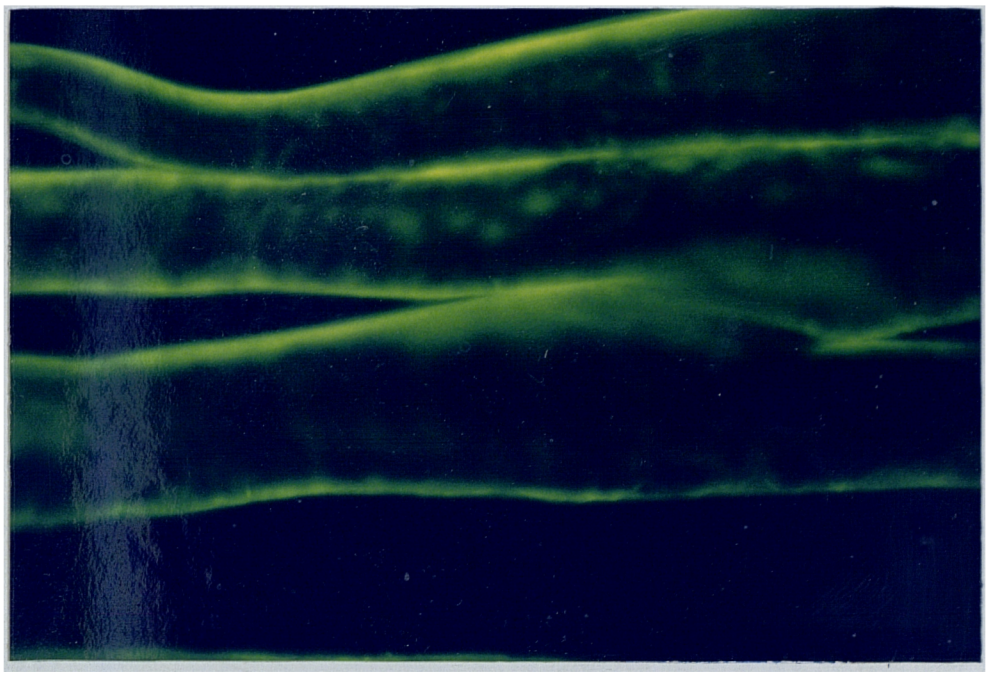


Fig. 2.17a

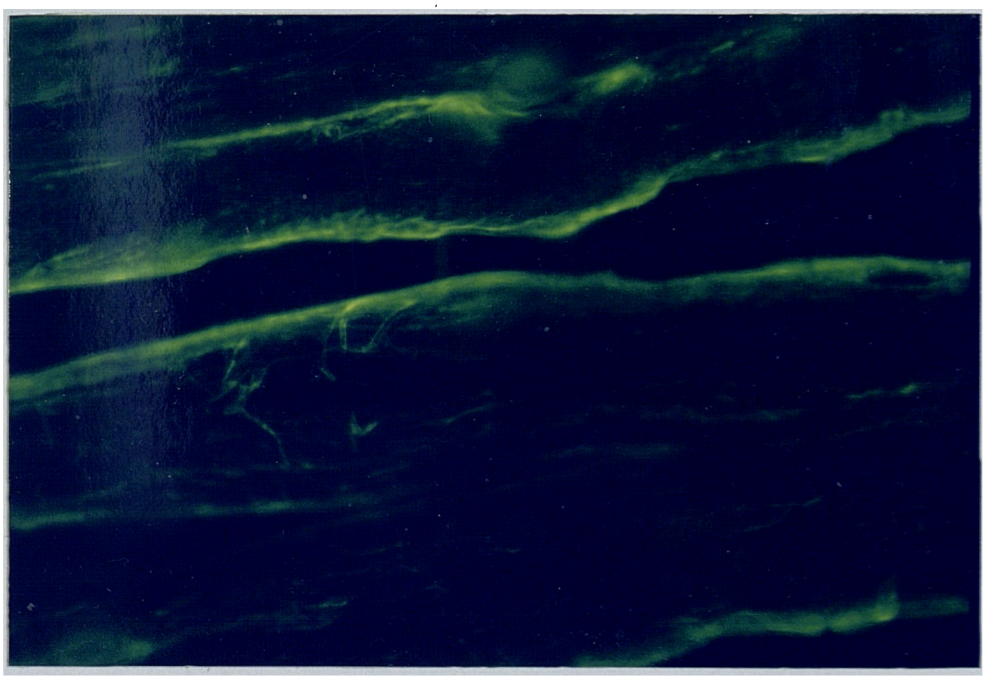


Fig. 2.17b

Fig. 2.17a & b Type III collagen labelled with a fluorescently tagged antibody on longitudinal frozen sections of (a) SDFT (x280) and (b) DDFT. (x110)

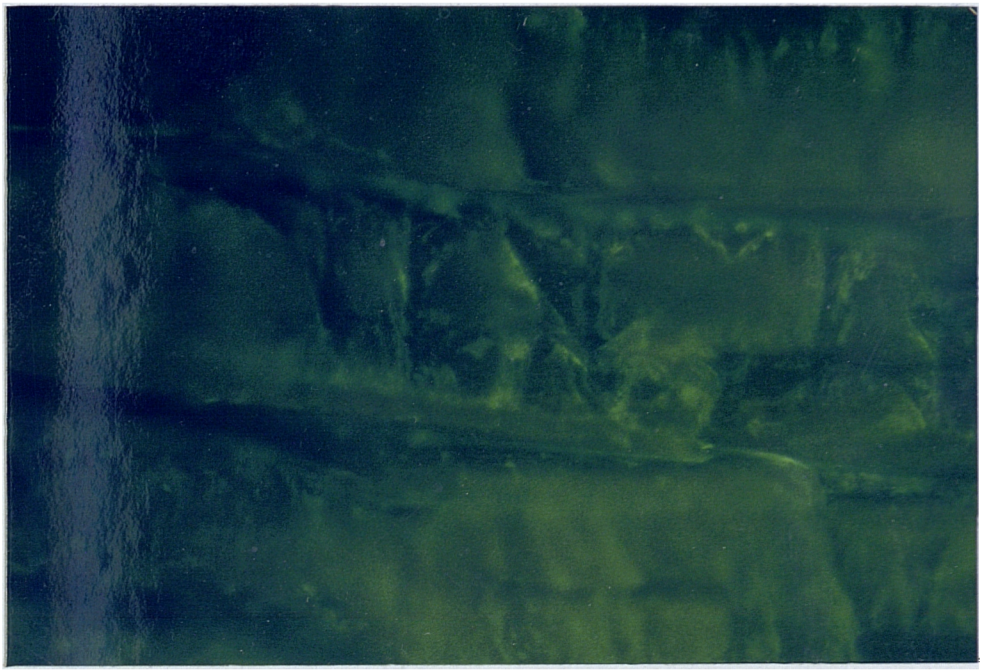


Fig. 2.17c Type I collagen labelled with a fluorescently tagged antibody on a longitudinal frozen section of SDFT. (x280)

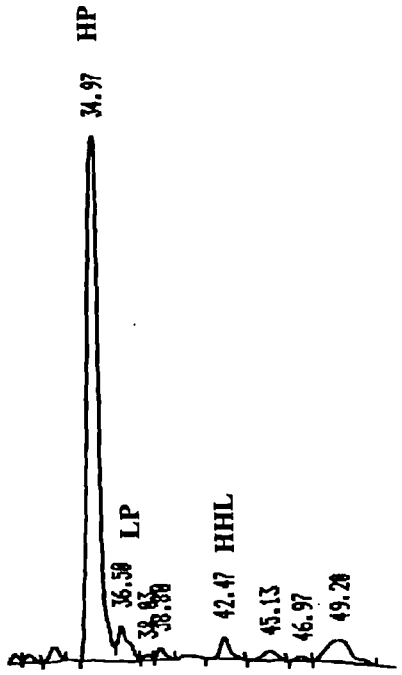


Fig. 2.18 Elution profile of collagen crosslinking amino acids from equine SDFT (group 3). HP (hyroxylysylpyridinoline), LP (lysylpyridinoline), HHL (histinohydroxylysinonorleucine).

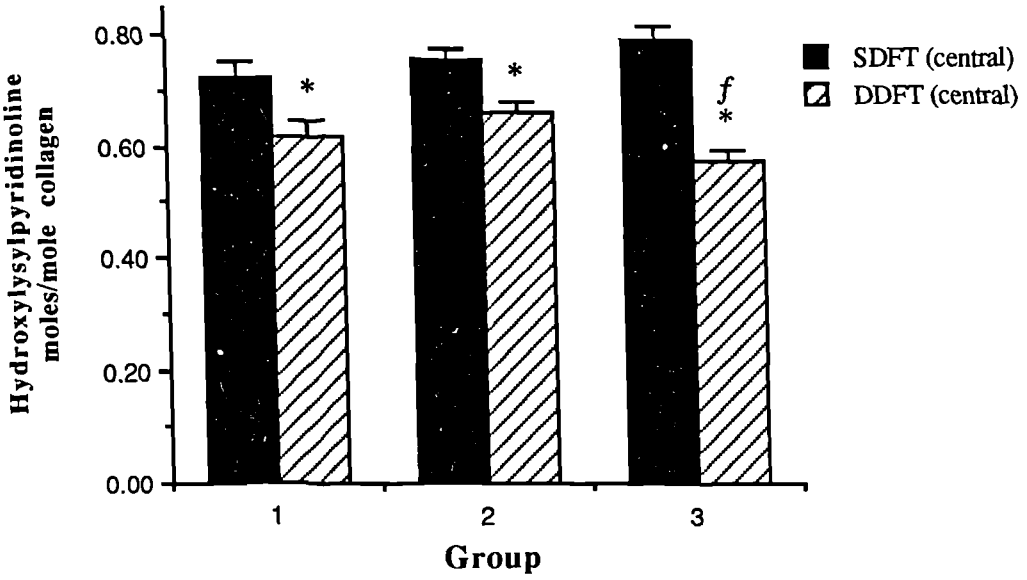


Fig. 2.19 Hydroxylysylpyridinoline crosslinks in equine flexor tendons. Group 1 = young horses (n = 5), group 2 = old un-exercised horses (n = 6) and group 3 = old exercised horses (n = 6). * denotes a significant difference relative to the SDFT and *f* a significant difference relative to group 2 tendons.

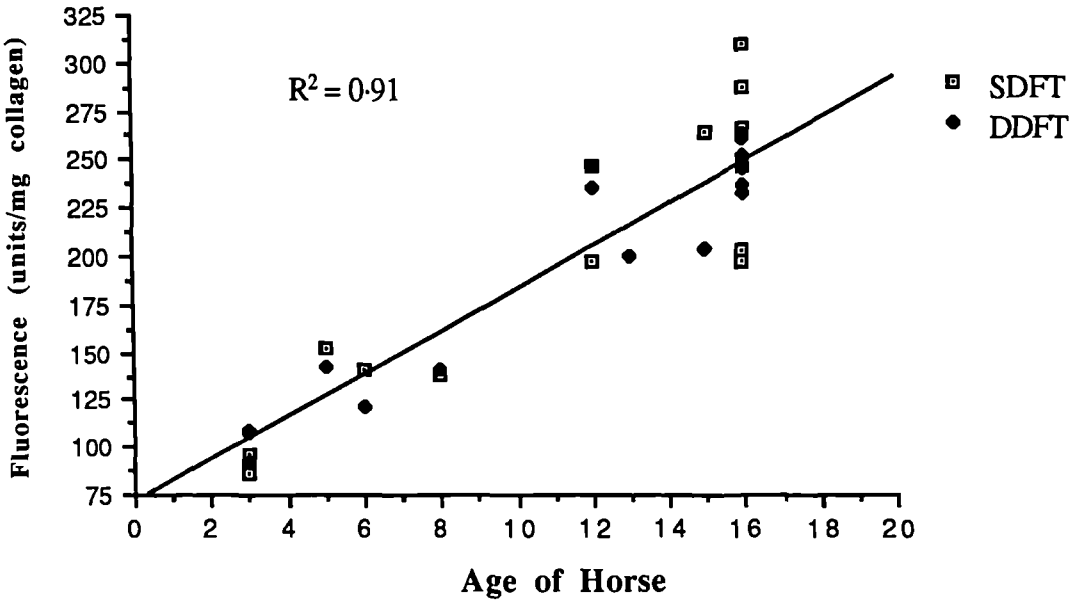


Fig. 2.20 Collagen-linked fluorescence in equine flexor tendons as a function of age. Points represent the average of the values obtained for central and peripheral zone tissue for each horse.

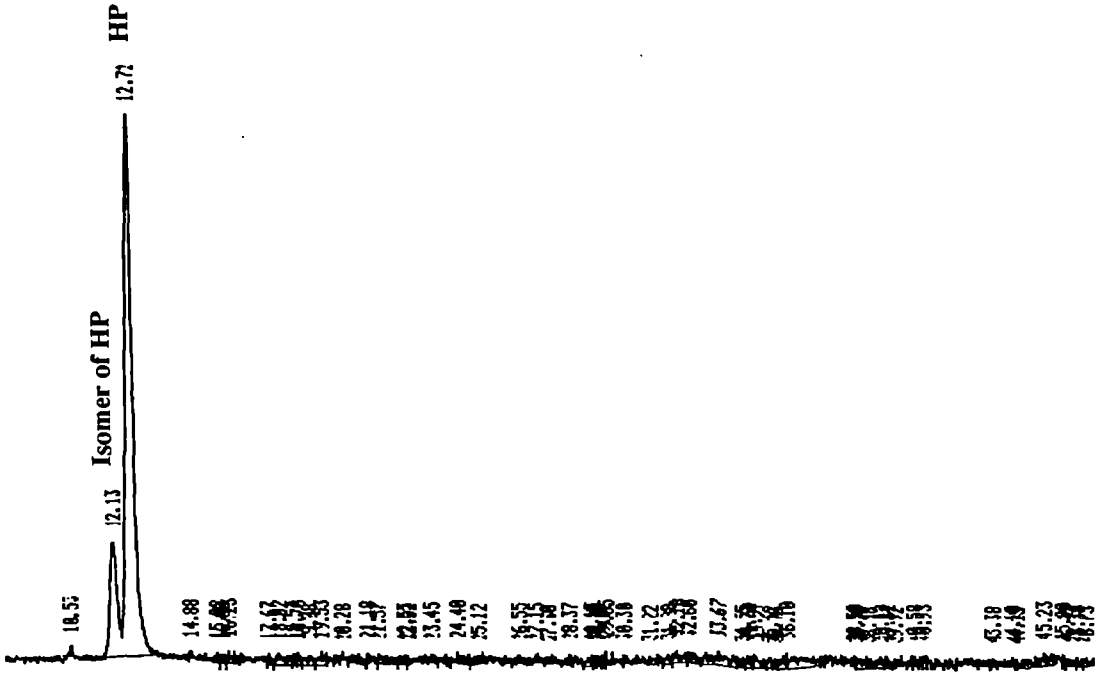


Fig. 2.21 HPLC elution profile of equine SDFT separated for glycosylated crosslink analysis. HP, hydroxylysylpyridinoline.

Table 2.1 Summary of equine SDFT composition. Group 1 = young horses (n = 5), group 2 = old un-exercised horses (n = 6) and group 3 = old exercised horses (n = 6). * denotes a significant difference relative to the DDFT, † a significant difference relative to the young group of horses and ¥ a significant difference relative to the peripheral tissue.

	SDFT					
	central			peripheral		
	group 1	group 2	group 3	group 1	group 2	group 3
% water content	65·1 ± 0·9 *	65·7 ± 0·8 *	67·3 ± 0·8 *	66·5 ± 0·9	67·2 ± 0·8	66·8 ± 0·8
DNA (μg/mg)	0·58 ± 0·09	0·63 ± 0·06 *		0·78 ± 0·09 *	0·51 ± 0·06 * †	
GAG (μg/mg)	10· 89 ± 1·78	9·48 ± 1·15 *		10·16 ± 1·78	7·79 ± 1·15 *	
% collagen content	77 ± 3	71 ± 3	80 ± 3	78 ± 3	77 ± 3	69 ± 3
% type III collagen	13·2 ± 2·5 *	15·7 ± 1·6 * ¥		10·5 ± 2·5	8·0 ± 1·6 *	
pyridinoline (mol/mol collagen)	0·72 ± 0·03 *	0·76 ± 0·02 *	0·80 ± 0·02 *	—	—	—
collagen-linked fluorescence	107 ± 20	229 ± 13 †		120 ± 20	234 ± 13 †	

Table 2.2 Summary of equine DDFT composition. Group 1 = young horses (n = 5), group 2 = old un-exercised horses (n = 6) and group 3 = old exercised horses (n = 6). * denotes a significant difference relative to the SDFT, † a significant difference relative to the young group of horses, ¥ a significant difference relative to the peripheral tissue and *f* a significant difference relative to old un-exercised horses.

	DDFT					
	central			peripheral		
	group 1	group 2	group 3	group 1	group 2	group 3
% water content	62·0 ± 0·9 * ¥	62·8 ± 0·8 * ¥	62·7 ± 0·8 * ¥	66·1 ± 0·9	67·8 ± 0·8	66·9 ± 0·8
DNA (μg/mg)	0·43 ± 0·09	0·27 ± 0·06 * †		0·39 ± 0·09 *	0·32 ± 0·06 *	
GAG (μg/mg)	13·64 ± 1·78	12·16 ± 1·15 *		13·15 ± 1·78	11·86 ± 1·15 *	
% collagen content	77 ± 3	72 ± 3	78 ± 3	72 ± 3	79 ± 3	74 ± 3
% type III collagen	4·5 ± 2·5 *	3·6 ± 1·6 *		6·7 ± 2·5	5·3 ± 1·6 *	
pyridinoline (mol/mol collagen)	0·62 ± 0·03 *	0·66 ± 0·02 *	0·57 ± 0·02 * *f*	—	—	—
collagen-linked fluorescence	112 ± 20	226 ± 13 †		116 ± 20	235 ± 13 †	

2.4 Discussion

Comparison of extracellular matrix composition of SDFT and DDFT

Considerable differences in cellularity and extracellular matrix composition were found between superficial and deep digital flexor tendons. Although percentage collagen content was the same for both tendons there were substantial differences in the collagen type and organisation. These differences can be related to the mechanical rôle of the tendon *in vivo*. Smaller diameter fibrils in the SDFT with respect to the DDFT supports the idea that this may be an adaptation to withstand creep (Parry *et al.*, 1978b). A greater number of smaller fibrils increases the potential for interfibrillar crosslinks and this may enable the SDFT, which experiences high strains at the gallop (Wilson, 1991), to return to its original length following elongation.

Type III collagen has been implicated in the control of fibril diameters; its presence limiting the radial growth of type I collagen fibrils (Fleischmajer *et al.*, 1981). In accordance, the levels of type III collagen were higher in the SDFTs than the DDFTs. High levels of type III collagen are associated with a greater degree of tissue elasticity and flexibility, as for example in the aorta (Farquharson & Robins, 1989). This is not likely to be a property of the type III molecule itself but rather due to its effect on collagen fibre organisation. As shown in the immunohistochemical localisation study, type III collagen predominated in the endotenon; the intratendinous membrane surrounding the subfascicular unit. Higher ratios of type III collagen in the SDFT may therefore reflect a smaller subfascicular unit in this tendon compared to the DDFT. Accurate measurement of subfascicular diameters was not possible in this study as longitudinal sections of tendon were used. Transverse sections, while ideal for such measurements, are difficult to cut using this method due to the fibrous nature of the tendon. Type III collagen fluorescence was however continuous across the fibre bundle and increased levels of type III collagen in the SDFT may therefore reflect an increased number of smaller type I collagen fibrils with type III collagen on their surface or an increased number of type III collagen fibrils. Localisation of collagen types at a fibrillar level may be possible using immunogold antibody labelling (Beesley, 1987); development of this technique is necessary to fully understand the significance of a higher type III collagen content in equine SDFT compared to DDFT.

High levels of the mature crosslink, hydroxylysylpyridinoline, have been associated with tissues subjected to high stresses and strains (Vogel & Koob, 1989) and again the higher levels present in SDFT compared to DDFT provides further evidence that a structure function relationship exists as proposed above. The potential for pyridinoline crosslink formation depends on the level of hydroxylation of the telopeptide lysine residues and their subsequent conversion to lysine aldehydes (Last *et*

al., 1990). Whether the differences in pyridinoline levels can be attributed to differing degrees of telopeptide hydroxylysine aldehyde formation is not known.

One of the factors influencing the degree of thermal stability of the collagen molecule, which may be of particular importance in the equine SDFT (see chapter 7), is the extent of hydroxylation of proline residues (Berg & Prockop, 1973); no difference however was observed in this parameter between SDF and DDF tendons. Similarly, total lysine residue hydroxylation, which has been found to increase in some pathological conditions e.g. osteoporosis (Bailey *et al.*, 1992), showed no difference between tendons. The minor differences observed in the amino acid residues serine, glutamate and isoleucine are probably due to differences in non-collagenous protein components such as proteoglycans and intracellular proteins.

Proteoglycans and their associated carbohydrate sidechains, have also been implicated in control of fibril diameters (Scott, 1986) and both the amount and type of GAG differed between the SDFT and DDFT. Chondroitin sulphate, which formed a similar proportion of the total GAG in both tendons, is the predominant GAG in cartilage and has an interfibrillar distribution acting mainly in a space filling capacity (Scott, 1986). Dermatan sulphate, a fibril associated GAG, is thought to limit radial growth of fibrils (Vogel *et al.*, 1984) and is present in higher amounts in the region of tendon subjected only to tension (Gillard *et al.*, 1977). As might therefore be predicted, dermatan sulphate was present in higher amounts in the tendon with smaller diameter fibrils (SDFT). Keratan sulphate, which is also fibril associated (Scott, 1991), and present in cartilage, formed a higher proportion of GAGs in the DDFT. It is not clear whether GAG composition controls collagen organisation or whether differences are secondary to a difference in fibrillar arrangement and this therefore represents an important area for future studies. Glycosaminoglycans are, however, turned over by the cells at a faster rate than the collagen component (Abrahamsson *et al.*, 1991) and therefore changes in GAG composition may precede a change in fibril formation.

Higher cellularity in the SDFT compared to the DDFT suggests that SDFT is a more metabolically active tissue. Similar levels of collagen-linked fluorescence in the SDFT and DDFT imply that there is no difference in collagen turnover rate but there may however be differences in the turnover rates of non-collagenous components. Synthesis of non-collagenous matrix components such as proteoglycans could be measured *in vitro* using explants of tissue from the SDFT and DDFT. These results indicate that differences exist between the SDFT and DDFT which are an adaptation to their different mechanical rôles.

Age related changes in the extracellular matrix of SDFT and DDFT

Collagen-linked fluorescence, in both the SDFT and DDFT, was time related and showed a clear and well correlated increase with age. All proteins within the body are susceptible to non-enzymatic glycosylation but those that have a relatively long half-life, such as collagen, are particularly prone to high levels of glycosylation (Reiser, 1990). Glycosylated residues may undergo a series of reactions resulting in the formation of fluorescent adducts and crosslinks known as advanced glycosylation end products (AGE). Pentosidine, an AGE product which has been detected in human extracellular matrix, could not be detected in any of the equine tendon samples and neither were any other unidentified peaks detected. This may be due to fluorescent end products not taking part in intermolecular crosslinking. AGE products are known to result in an increase in tissue stiffness (Rolandi *et al.*, 1991); therefore if the tendon is subjected to the same stress a lower strain and diminution in energy storage will result. It may be that in the equine flexor tendons, where mechanical properties are crucial to physiological function, AGE product crosslinks are not formed thus preventing a change in mechanical properties with increasing age. Factors affecting protein glycosylation include blood glucose concentration, which is similar in both man and horse (5 mM), blood supply to the particular tissue and collagen turnover rate. Recent studies have shown that formation of AGE from glycosylated residues depends upon an oxidative environment and therefore low tissue oxygen tension may have an inhibiting effect on formation of pentosidine and other glycosylated crosslinks (Fu *et al.*, 1992). Prevention of AGE product formation in equine flexor tendons is not likely to be due to high collagen turnover rates but rather to the poor blood supply to the tendon.

Cellularity in the DDFT decreased on ageing particularly in the central region and probable reflects the low matrix turnover and thus metabolic demand in this tendon. In the SDFT however ageing did not result in a decreased cellularity suggesting in this tendon there is need for a higher metabolic capacity to maintain the extracellular matrix in a form able to carry out its mechanical rôle.

Exercise related changes in the extracellular matrix of SDFT and DDFT

No differences in the above factors were apparent in the SDFT between the trained and untrained group of horses. It may be that the SDFT does not undergo a change in composition in response to training; the SDFT being already be at its optimum to carry out its function. Any increase in the stiffness of the tendon would result in a reduction in its effectiveness to act as an elastic energy store. Alternatively, the failure to find any change with exercise, either that which might be expected to

increase the tensile strength or a degenerative change, may be due to an inaccurate assignment of horses to each of the groups. A history and description of the type of work undertaken by each horse was taken from the owner and this area is a possible source of error. Personal opinions differ on what may be described as light work only and what constitutes a vigorous training programme. Other factors such as conformation, fitness and health may also affect the load flexor tendons are subjected to during exercise and may therefore mask any differences.

Comparison of the central and peripheral zone of SDFT and DDFT

Water content in the DDFT was significantly lower in the central zone compared to the peripheral zone of the tendon. A reduction in water content increases the stiffness of a tissue (Dorrington, 1980) but the small differences found here are unlikely to be of any functional importance. No significant differences in composition were seen between central and peripheral regions of the SDFT in the young group of horses. These data provide no evidence for a difference in mechanical strength between the two zones or a predisposition of the central region to overload. In the old group of SDFTs however, the central zone had a significantly higher proportion of type III collagen than the peripheral zone of the tendon. Type III collagen, through its effect on matrix organisation, will reduce the material properties of this zone of the tendon resulting in a central core weakness. If the equine SDFT is assumed to be mechanically homogeneous in young horses but displays different mechanical properties between central and peripheral zones in old horses (Wilmink *et al.*, 1992), this suggests that such a mechanical difference results from biochemical or organisational changes within the matrix of the tendon. These changes presumably reflect differences in cellular synthetic activity in the different zones of the tendon.

Chapter Three

EXTRACELLULAR MATRIX COMPOSITION OF DEGENERATED EQUINE SUPERFICIAL DIGITAL FLEXOR TENDONS

3.1 Introduction

It was first suggested by Forsell (1952) that an intra-tendinous degenerative change precedes rupture. Changes have been found within the central core of equine superficial digital flexor tendon (SDFT) from horses that were still in active racing (Webbon, 1977). Macroscopic examination of a transverse section from these tendons showed a pink or purple core. To date no quantitative measurements of extracellular matrix components accompanying the observed macroscopic changes have been made. Similarly, in human Achilles tendon, rupture is thought to be preceded by a degenerative change (Arner *et al.*, 1959). Several histological studies have been carried out on human Achilles tendon immediately post rupture; hypoxic changes to tenocytes (Józsa *et al.*, 1982) and structural alterations to collagen fibrils and fibres were observed (Józsa *et al.*, 1984 & 1989). The most frequent alterations were longitudinal splitting, disintegration and angulation of collagen fibrils and a decrease in the average collagen fibre diameter. It has been shown, however, that tendon specimens strained to failure *in vitro* display wide-spread damage such that every fibril is disintegrated prior to tendon rupture (Kastelic & Baer, 1980). Changes to collagen fibrils and fibres in this study, therefore, may occur as a direct result of the load which caused the tendon to fail rather than representing a true degenerative change. Quantitative measurements of individual matrix components were not made in these studies and therefore the significance of these findings remains unclear.

If the observed macroscopic changes in some equine SDFTs represent a degenerative change that predisposes to tendon rupture they must be accompanied by changes to the extracellular matrix resulting in a mechanically weaker tendon. Alteration to the extracellular matrix may be due to disruption of the "normal" matrix components caused by repetitive loading cycles accompanied by an inability of tenocytes to repair such microdamage. Alternatively, degeneration may be due to either an increase in matrix degradation and/or a loss of synthetic capacity by tenocytes resulting in a decreased collagen content. Finally, degenerative change may be due to a change in cell function and alteration of matrix component type and organisation.

Hypothesis

The macroscopic appearance of degenerated SDFTs is accompanied by changes in extracellular matrix composition which result in a mechanically weaker tendon.

Objectives

1. To quantify extracellular matrix components in the central core of degenerated SDFTs.

2. To compare the central and peripheral zone of degenerated SDFTs and compare with the same zone in tendons with a normal appearance.

Experimental design

In this part of the study quantitative measurements of matrix components will be made in the central region of degenerated SDFTs. A comparison with the peripheral region of the same tendon and also with central and peripheral zone tissue from "normal" tendons will be made. Data will demonstrate whether macroscopic appearance is associated with a weakening of the extracellular matrix of the tendon. Data will also be used in following chapters where cells are metabolically stressed *in vitro*; the response of the cells can then be compared to what is known to happen to degenerated tendons *in vivo*.

3.2 Methods

Tissue collection

Superficial digital flexor tendons were collected from the forelimbs of half- to full-Thoroughbred horses slaughtered at a local abattoir, for reasons other than tendon injury. A brief history and age of the animal was obtained from the owners and ages verified by examination of the teeth (see appendix 2). Tendons were palpated pre mortem in the standing horse and again on removal from the animal and any showing signs of thickening were excluded from the study.

A sample of tissue (approx. 4 cm length) was taken from the mid-metacarpal region of the SDFT (see fig. 2.1). Tendon samples were cut transversely (into 2 x 2 cm lengths) and examined carefully for signs of a central core discolouration. Those tendons with a clearly and well defined reddish central core (fig. 3.1) were placed into the group designated "degenerated" tendons (n = 6, mean age 9·0 yr). A group of "normal" tendons with a homogeneous pinkish yellow colour in transverse section (fig. 3.2) were also selected (n = 6, mean age 12·7 yr). The group of degenerated tendons were analysed along side the normal group (in addition to comparison with values obtained in the previous chapter) in case of variation between assay runs. One 2 cm length of each tendon was separated into central and peripheral zone tissue (see fig. 2.2), and stored at -20°C prior to biochemical analysis. The other 2 cm section was kept for cutting frozen sections (stored at -20°C).

Biochemical analyses

The following parameters were measured in central and peripheral zone tissue from each of the tendons; water content, deoxyribonucleic acid (DNA), glycosaminoglycans (GAGs), collagen content, proline and lysine hydroxylation, type III collagen, collagen crosslinks, collagen-linked fluorescence and glycosylated crosslinks. In addition, type III collagen was localised on frozen sections using immunohistochemical techniques. The methods used were identical to those in chapter 2, as are the units of measurement.

Fig. 3.1 Transverse section at mid-metacarpal level through equine SDFT showing a central core degeneration. (x4·5)

Fig. 3.2 Transverse section at mid-metacarpal level through a normal equine SDFT. (x4·5)

Statistical analysis

Statistical significance was evaluated using a mixed model ANOVA test where horse was a random effect and tendon group (normal or degenerated) and zone (central or peripheral) were fixed effects. The level of significance was taken as $p \leq 0{\cdot}05$. Data are presented as mean ± S.E.M.

3.3 Results

The results from analysis of degenerated equine SDFTs are summarised in table 3.1.

Water content

Water content did not differ significantly between central and peripheral zone tissue for either group or between the normal and degenerated groups of tendons (fig. 3.3). The values obtained for the normal group of tendons were similar to those obtained in the previous chapter.

DNA content

Both central and peripheral zones of degenerated tendons had an increased DNA content (fig. 3.4) relative to normal tendons, indicating a higher cellularity, although the difference was not significant. Values obtained for normal tendons were not significantly different to those obtained in the previous chapter.

Total sulphated glycosaminoglycan content

The central zone of degenerated tendons had significantly higher levels of chondroitin sulphate equivalent GAGs relative to the peripheral zone within the same tendon ($p < 0{\cdot}002$) and to the central zone of the normal tendons ($p < 0{\cdot}002$) (fig. 3.5). Values for the normal tendons were not significantly different to those obtained in the previous chapter.

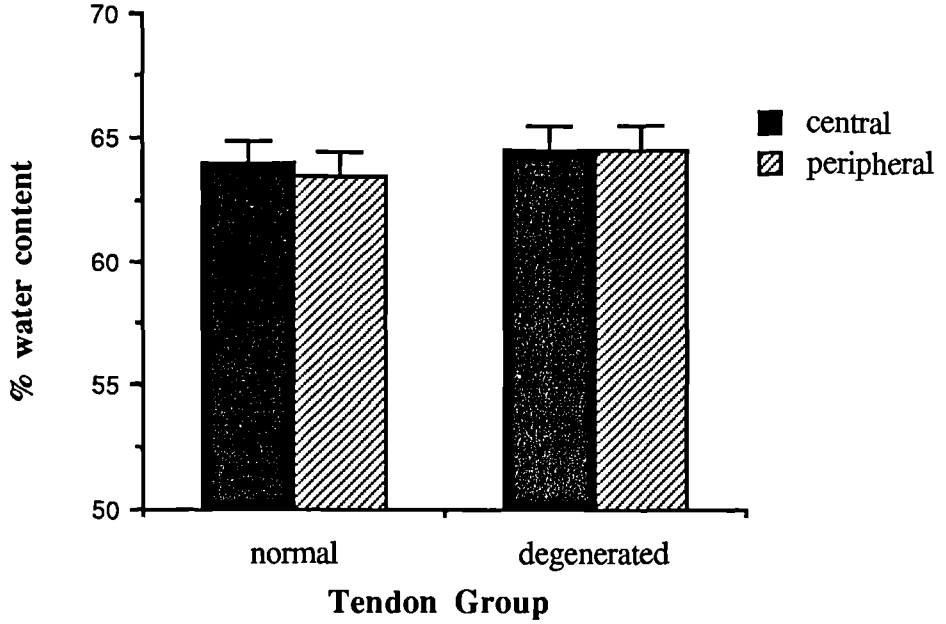


Fig. 3.3 Percentage water content in normal and degenerated equine SDFT. n = 6 for each group.

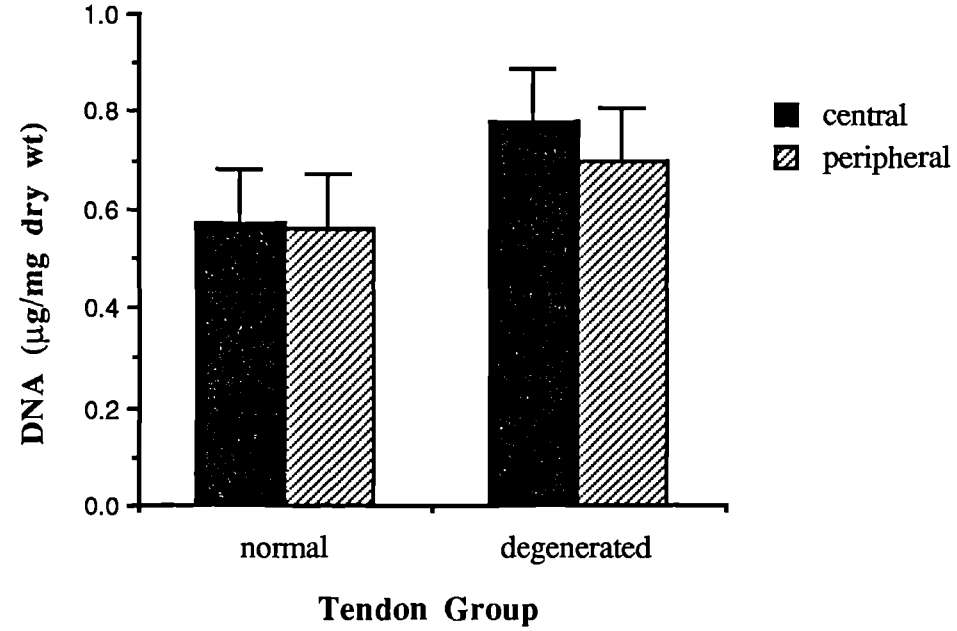


Fig. 3.4 DNA content in normal and degenerated equine SDFT. n = 6 for each group.

Collagen content

No significant differences were observed between central and peripheral zones for either group of tendons or between the normal and degenerated groups (fig. 3.6). Values for the normal group of tendons were not significantly different to those obtained in the previous chapter.

Percentage hydroxylation of proline and lysine residues

The percentage hydroxylation of proline and lysine residues (table 3.1) did not differ significantly between groups or between central and peripheral tissue within a group.

Percentage type III collagen

Type III collagen formed a significantly higher proportion of total collagen in the central zone tissue compared to the peripheral zone in both groups of tendons ($p < 0{\cdot}025$) (fig. 3.7). The highest value for type III collagen content ($17{\cdot}2\% \pm 1{\cdot}9$) was obtained for the central tissue in the group of degenerated tendons. Figure 3.8 shows cyanogen bromide (CNBr) peptides from the central and peripheral zones of normal and degenerated equine SDFTs separated by electrophoresis on a polyacrylamide gel.

Immunohistochemical localisation of type III collagen

Examination under the microscope of frozen sections from the group of normal tendons, labelled with fluorescein isothiocyanate tagged anti-type III collagen antibody, showed a brightly stained region at the edges of the subfascicular unit (fig. 3.9 a). Similar observations were made in SDFTs and deep digital flexor tendons (DDFTs) in the previous chapter and demonstrate that type III collagen is located predominantly in the endotenon membrane around the collagen fibre bundles. Degenerated tendons also showed a brightly stained region at the edge of fibre bundles. In addition, degenerated tendons showed a much brighter stain within the subfascicular unit suggesting an increased deposition of type III collagen fibrils within the actual collagen fibres (fig. 3.9 b). In some cases, degenerated tendons showed areas where type III collagen staining was associated with a loss of subfascicular structure (fig. 3.9 c).

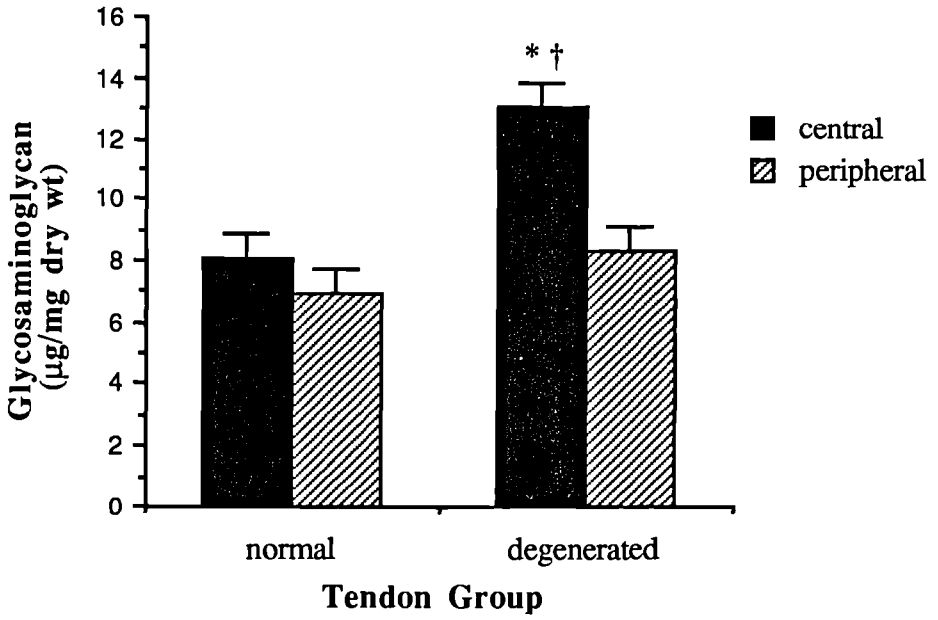


Fig. 3.5 Total sulphated chondroitin sulphate equivalent glycosaminoglycan content in normal and degenerated equine SDFT. n = 6 for each group. * denotes a significant difference relative to the peripheral tissue within the same group and † a significant difference relative to the same zone in the normal group of tendons.

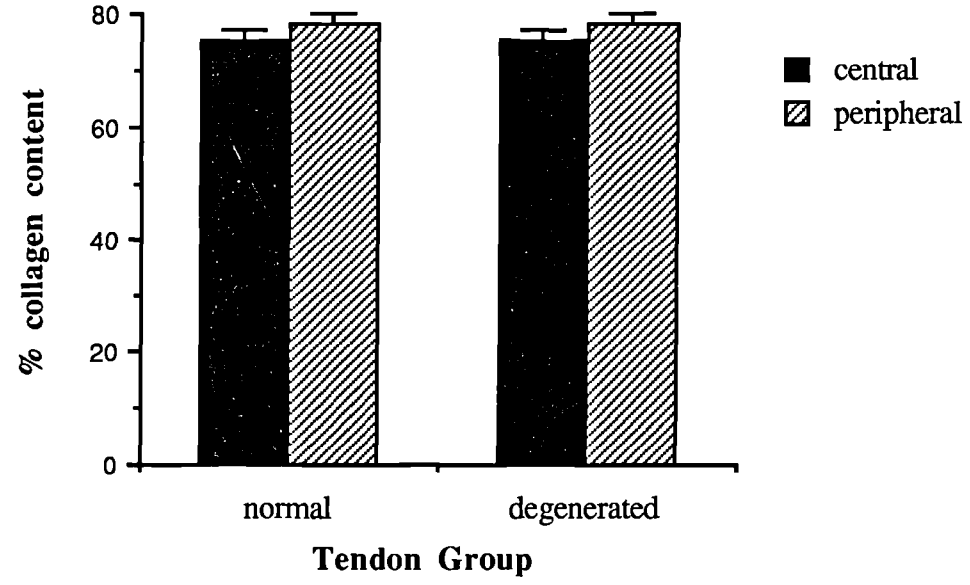


Fig. 3.6 Percentage collagen content in normal and degenerated equine SDFT. n = 6 for each group.

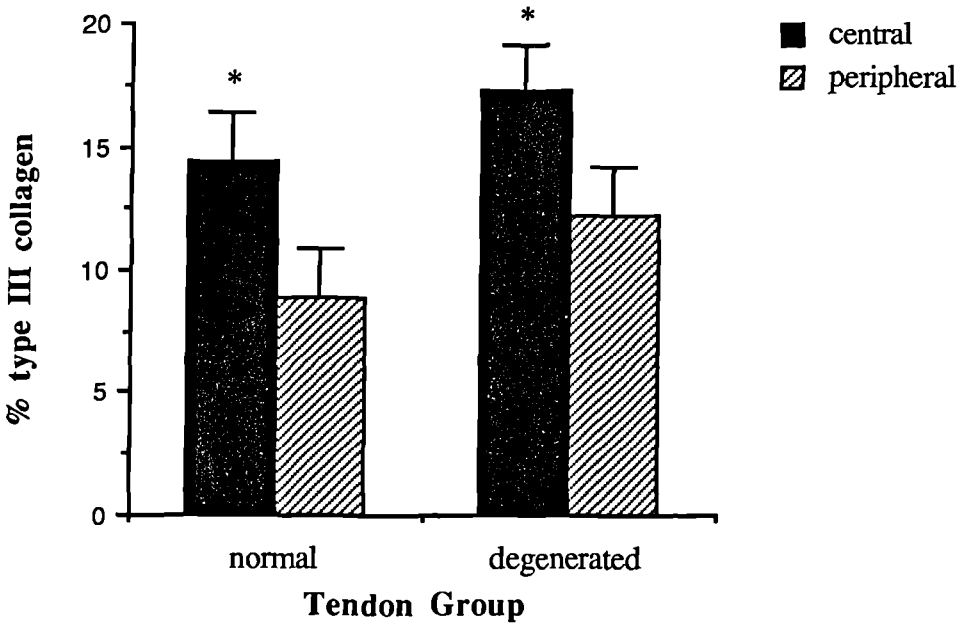


Fig. 3.7 Percentage type III collagen of type I + III collagen in normal and degenerated equine SDFT. n = 6 for each group. * denotes a significant difference relative to the peripheral tissue within the same group.

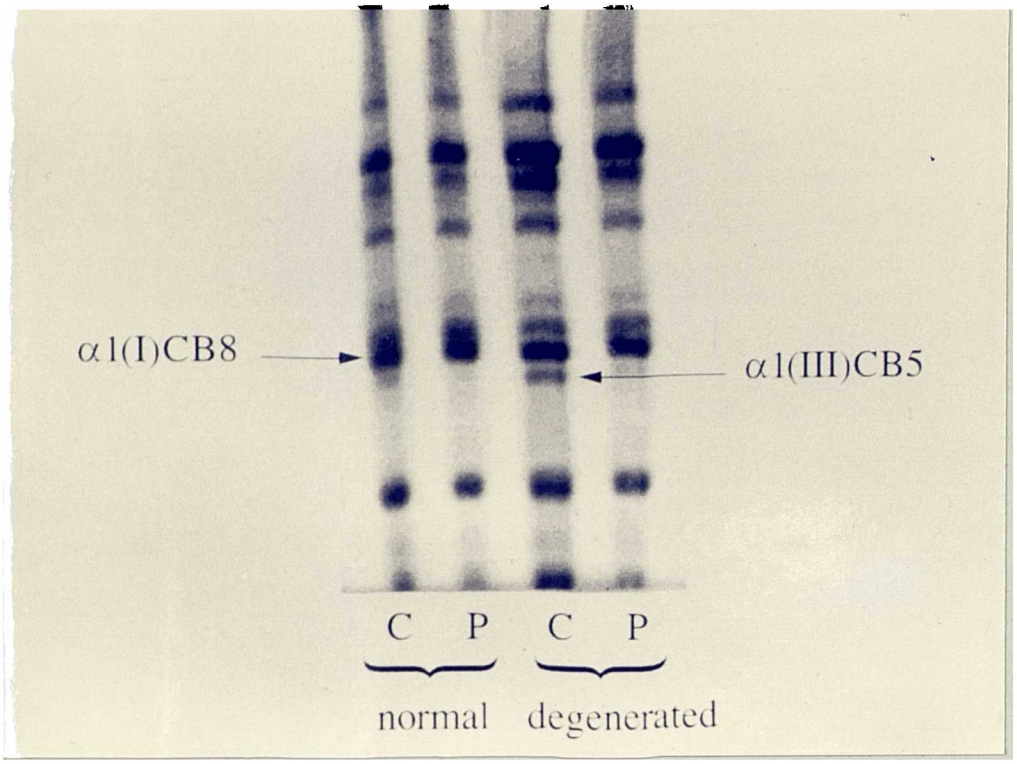


Fig. 3.8 Separation of CNBr peptides from central and peripheral zone tissue of normal and degenerated equine SDFT on a SDS-polyacrylamide gel by electrophoresis.

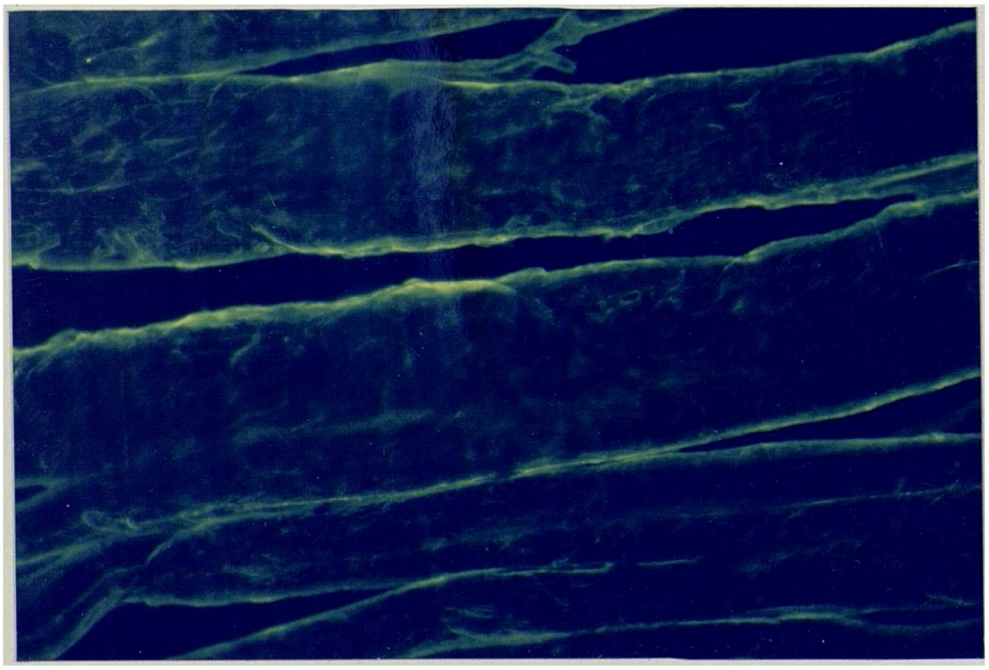


Fig. 3.9a Type III collagen labelled with a fluorescently tagged antibody on a longitudinal frozen section of normal equine SDFT. (x110)

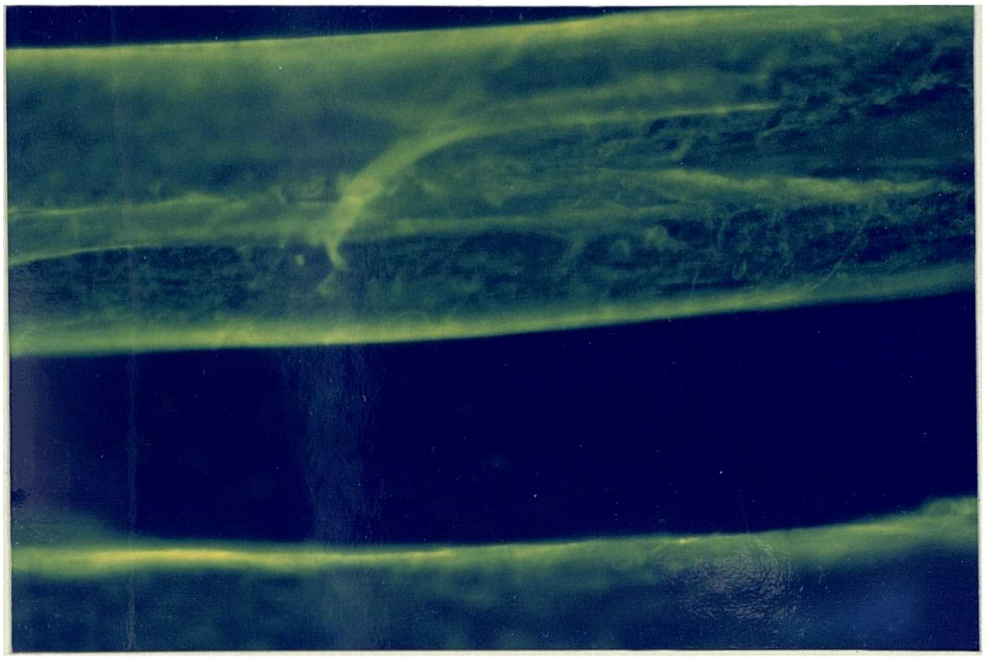


Fig. 3.9b

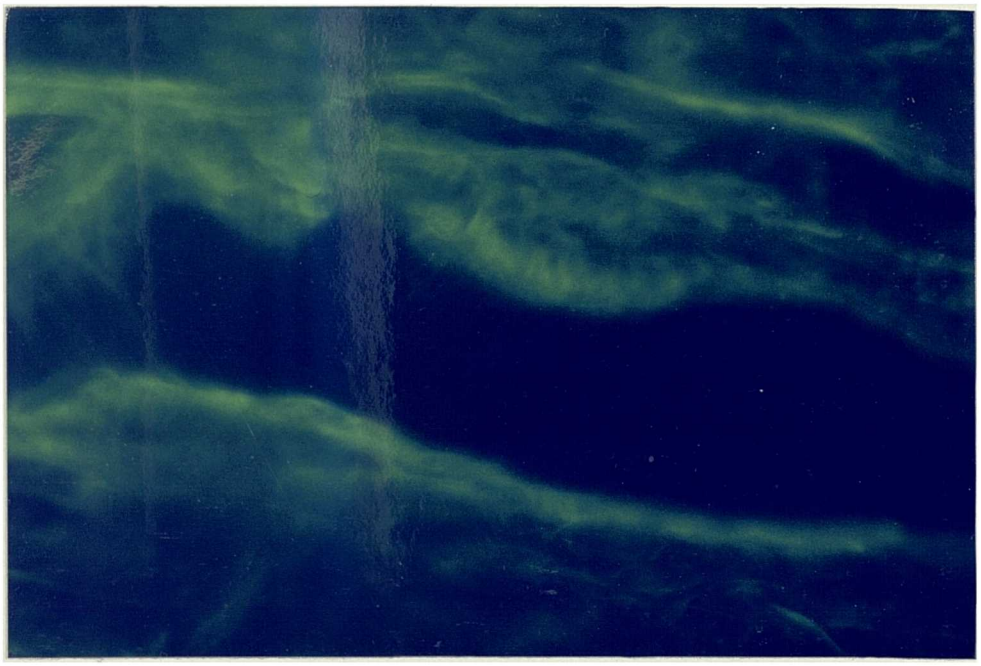


Fig. 3.9c

Fig. 3.9b & c Type III collagen labelled with a fluorescently tagged antibody on longitudinal frozen sections of degenerated equine SDFT showing brightly stained subfascicles (b) and loss of subfascicular structure (c). (x280)

Crosslink analysis

As in the previous chapter, the predominant crosslink present in both groups of tendons was hydroxylysylpyridinoline (HP). Reducible crosslinks and the mature crosslinks lysylpyridinoline (LP) and histidinohydroxylysinonorleucine (HHL) were present in both groups at very low levels. The level of HP was not significantly different between central and peripheral tissue within either group or between groups (fig. 3.10).

Collagen-linked fluorescence

The central zone tissue from the degenerated group of tendons showed a significant decrease in fluorescence compared to the peripheral zone tissue ($p < 0{\cdot}01$). There was no significant difference between zones in the normal group of tendons. In the previous chapter the level of fluorescence showed a tight correlation with the age of the horse, therefore, as there is a wide variation of age within the two groups, the value obtained for central tissue was divided by that obtained for the peripheral tissue and a plot of this ratio is shown in figure 3.11.

Glycosylated crosslinks

Pentosidine was not detected in any of the samples from either group of tendons. As in the previous chapter, no other fluorescent crosslinks formed by non-enzymatic glycosylation could be detected either.

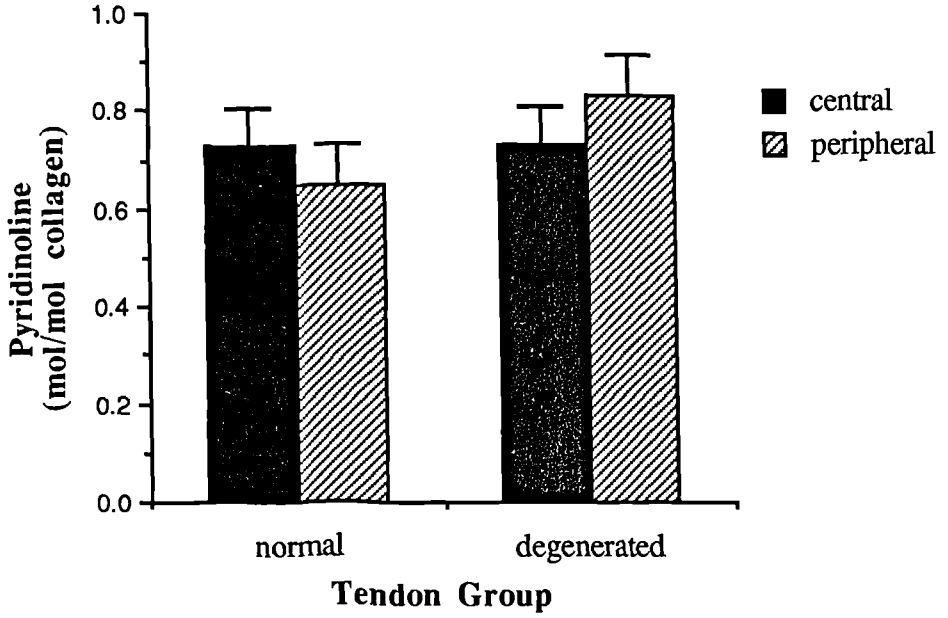


Fig. 3.10 Hydroxylysylpyridinoline crosslinks in normal and degenerated equine SDFT. n = 6 for each group.

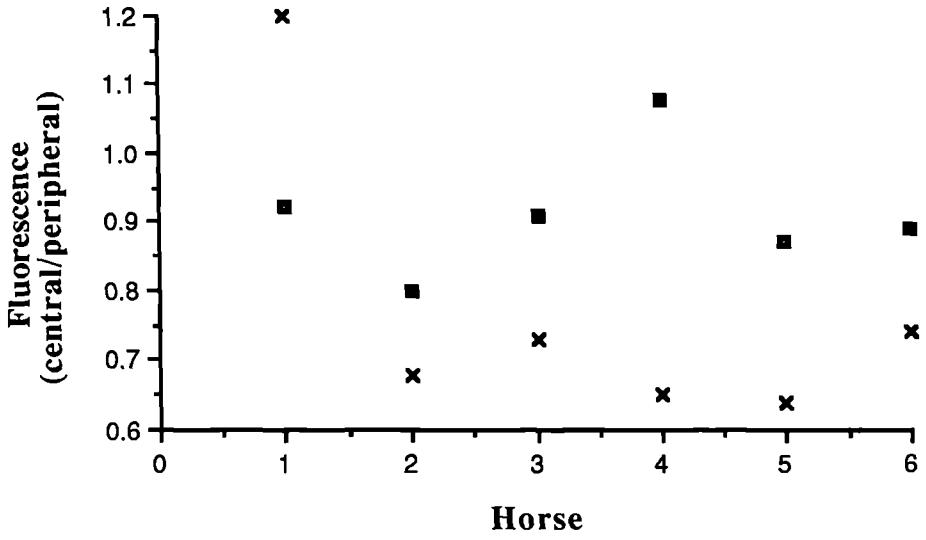


Fig. 3.11 Ratio of central to peripheral zone tissue collagen-linked fluorescence in normal and degenerated equine SDFT. (• = normal tendons, x = degenerated tendons)

Table 3.1 Summary of normal and degenerated equine SDFT composition. * denotes a significant difference relative to the peripheral zone tissue and † a significant difference relative to the same zone in the normal group of tendons.

	SDFT			
	normal		degenerated	
	central	peripheral	central	peripheral
% water content	64·0 ± 0·9	63·5 ± 0·9	64·6 ± 0·9	64·6 ± 0·9
DNA (μg/mg dry wt)	0·57 ± 0·11	0·56 ± 0·11	0·78 ± 0·11	0·70 ± 0·11
GAG (μg/mg dry wt)	8·08 ± 0·79	6·91 ± 0·79	13·10 ± 0·79 *†	8·38 ± 0·79
% collagen content	75 ± 2	78 ± 2	75 ± 2	78 ± 2
% hydroxylation of proline residues	43·5 ± 2·3	45·0 ± 2·1	41·0 ± 2·1	41·0 ± 1·9
% hydroxylation of lysine residues	24·4 ± 0·7	23·7 ± 0·7	25·4 ± 0·7	24·3 ± 0·7
% type III collagen	14·4 ± 1·9 *	8·9 ± 1·9	17·2 ± 1·9 *	12·2 ± 1·9
pyridinoline (mol/mol collagen)	0·73 ± 0·08	0·65 ± 0·08	0·73 ± 0·08	0·83 ± 0·08
collagen-linked fluorescence	172 ± 9	189 ± 9	139 ± 9 *	185 ± 9

3.4 Discussion

Tendons showing a macroscopic alteration to the central core, characterised by a reddish staining visible in transverse sections, but no gross increase in cross sectional area, showed a different matrix composition relative to the peripheral region of the same tendon and to a group of macroscopically normal tendons. The central core of degenerated tendons had increased total sulphated glycosaminoglycan contents, high levels of type III collagen, a decrease in collagen-linked fluorescence and high cellularity. In the analysis of SDFTs in chapter 2, the old group of horses had a significantly higher proportion of type III collagen in the central tissue compared to the peripheral tissue, while there was no difference in the young group of horses. In addition, although cellularity did not increase, SDFTs showed no decrease with ageing unlike the DDFTs. Thus, these tendons may also be undergoing early degenerative changes, which may occur prior to the appearance of the reddish staining in the central core.

Degenerative changes, if predisposing the tendon to injury, must be associated with a reduction in the mechanical strength of the tendon. A high type III collagen content may be responsible for this weakening, either through a disruption of subfascicular structure as in Dupuytren's disease (Bailey, 1990), or as a result of an increase in fine type III collagen fibrils within the collagen fibres. A change in the glycosaminoglycan content is likely to be associated with changes in matrix organisation and may result in a reduction of material properties. High cellularity alone would not be expected to influence the mechanical properties of the tendon directly, however, cell to cell signalling resulting in a change in cellular matrix metabolism may result in production of a weaker matrix.

In the light of these findings the proposed possible aetiologies of degenerative change introduced in section 3.1 can be examined. It appears unlikely that degenerative change can be attributed purely to direct damage caused by repetitive loading cycles, although there may be indirect effects (see chapters 6 & 7). This is because changes seem to be localised in the central core of the tendon. As there are no differences in matrix composition between central and peripheral zones of SDFT in young un-exercised horses (chapter 2) there is no reason to believe that central fibres would be preferentially loaded. Whilst repetitive loading may fragment type I collagen fibres this would not produce an increased type III collagen content. The lower collagen-linked fluorescence value for degenerated central core tissue suggests that there is an increase in collagen turnover and together with high cellularity indicate that cell activity and ability to repair microdamage is not impaired. Collagen content did not differ between normal and degenerated tendons and therefore the possibility that an increase in collagen degradation alone is responsible for degeneration can be discarded. As total

collagen content remained the same within the central and peripheral zones but the percentage of type III increased in the central tissue there must be an increase in collagen turnover i.e. both in degradation and synthesis of collagen. Levels of the crosslink hydroxylysylpyridinoline, were however, un-changed in both peripheral and central zone tissue of degenerated tendons and reducible crosslinks present at only low levels suggesting that increased collagen turnover is occurring over a longer time course than that which for example, occurs during repair following gross injury. It therefore seems likely that alterations to the central zone tissue, as seen in degenerated tendons, is due to a change in cell function.

The red staining visible in the central core of degenerated tendons may be due to hyperaemia or hypercapillarisation of this part of the tendon and may precede the other observed changes. Alternatively, and more likely, mechanical overload, due to a weakening of this part of the tendon, may result in damage to small capillaries and haemorrhage. Tissue damage often results in an inflammatory response manifested by a complex sequence of closely interrelated events. Swelling, heat and pain are symptoms associated with an inflammatory response, none of which appear to occur in these tendons at this stage, as damage is not clinically detectable. Similarly, in human Achilles tendon, degenerative changes have been observed in tendon tissue with no clinical history of inflammation or presence of inflammatory cell types (Puddu *et al.*, 1976). Therefore, active inflammation is not involved in this stage of the pathological process.

Thus in summary, degenerative changes are characterised by a change in cell function in the central core of the tendon resulting in increased collagen turnover, type III collagen deposition, and GAG synthesis. The stimulus for this change remains unclear, but may be due to a change in biochemical environment such as oxygen tension or hyperthermia (see chapters 6 & 7), as a result of repetitive loading cycles. Factors which may result in a change in cell matrix metabolism will be investigated in chapters 6 & 7. Conditions of interest are those which result in a stimulation of cellular activity, particularity type III collagen secretion and do not result in cell death.

Chapter Four

ISOLATION OF TENOCYTES AND INVESTIGATION OF ENERGY METABOLISM IN THESE CELLS

4.1 Introduction

The energy metabolism of tenocytes is not completely understood. Tendon, unlike muscle, does not require energy to perform an active mechanical rôle during locomotion (Evans & Barbenel, 1975); the passive mechanical properties of tendon are largely conferred by the extracellular matrix. The cells, however, are responsible for maintaining this matrix through the synthesis, assembly, deposition and organisation of extracellular matrix molecules (Doane & Birk, 1991) and thereby, the mechanical properties of the tendon (Fackelman, 1973). Thus, it is likely that the cells have an energy requirement for synthesis of matrix components. A number of early studies on rat tendons have suggested that collagen turnover in this structure is essentially zero in the mature animal (Neuberger *et al.*, 1951; Thompson & Ballou, 1956) and implied that tendon cells in mature tissue might be metabolically inert. However, it is now well accepted that collagen and other matrix proteins are continually being turned over, allbeit at a slow rate (Gerber *et al.*, 1960). Protein synthesis is an energetically costly process requiring the hydrolysis of one adenosine triphosphate (ATP) molecule for each amino acid added to the growing chain (Stryer, 1988). Energy is also required for intracellular movement of collagen and its secretion from the cell (Kruse & Bornstein, 1975). Few investigators have examined tenocyte energy metabolism (for a review see chapter one). The latest studies carried out by Landi *et al.* (1980a) and Floridi *et al.* (1981) suggest that mature tenocytes are completely anaerobic.

Hypoxia, resulting from poor blood supply to the tendon and its further compromise during mechanical loading, has been suggested as a possible cause of damage to the tendon (Smart *et al.*, 1980; Fackelman, 1973). It is therefore important to assess the effect of hypoxia on the tenocytes. To do this one must first know the relative contribution of anaerobic and aerobic metabolism to ATP production.

Hypothesis

Mature equine tendon fibroblasts from the superficial digital flexor tendon (SDFT) possess mitochondrial enzymes and therefore the potential for aerobic metabolism.

Objectives

1. To isolate intact viable cells from specific regions of equine SDFT.

2. To determine the likely rôles played by aerobic and anaerobic metabolism in the production of ATP by tenocytes.

Experimental design

It is difficult to study tenocytes in intact tendon due to their inaccessibility and whole tendon homogenates are impossible to obtain by mechanical means without damaging the cells. Thus, cell isolation by collagenase digestion was chosen for this part of the work to allow the free movement of metabolites to and from the cells. Isolation of cells by this method is commonly used in a variety of tissues such as heart, liver, kidney and adipose tissue (Elliot, 1979) and has also been used to release cells from embryonic chick tendons (Dehm & Prockop, 1971). Cells in mature tendons, however, are surrounded by a large amount of enzymaticaly resistant extracellular matrix making it much more difficult to release cells by this method and hence it has not been used previously to obtain mature tendon cells.

To assess the potential for oxidative metabolism in tenocytes, the maximal activities of key enzymes involved in glycolysis (lactate dehydrogenase) and mitochondrial respiration (citrate synthetase) were measured. Lactate dehydrogenase, a cytosolic enzyme, only takes part in the anaerobic metabolism of glucose. Citrate synthetase is found exclusively within the mitochondria and is a rate limiting enzyme in the aerobic metabolism of glucose. The reaction catalysed by citrate synthetase represents an important control point in the Krebs cycle. The activities of other mitochondrial enzymes (malate dehydrogenase and glutamate dehydrogenase) were measured to provide further evidence for the existence of mitochondria. As the central zone of the tendon is notable in being particularly susceptible to degeneration and subsequent injury (Fackelman, 1973; Webbon, 1977), cells from central and peripheral zones were separated. This allows exploration of the possibility that the central zone is metabolically inert and lacks "active" cells capable of matrix regeneration.

The presence of mitochondrial enzymes indicate the potential for aerobic metabolism of glucose but does not prove that this pathway is utilised for the generation of ATP. Therefore, the flux of glucose through glycolysis and the Krebs cycle was assessed directly, in intact cells by radioisotopic methods.

4.2 Methods

Isolation of cells

Superficial digital flexor tendons were collected at a local abattoir from half- to full-Thoroughbred horses with no known history of clinically detectable tendon injury, and transported to the laboratory on ice in sterile PBS (phosphate buffered saline). The un-sheathed region of tendon, from the distal reflection of the carpal synovial sheath to just above the metacarpo-phalangeal joint (approx. 15 cm), was selected and all loose connective tissue dissected away. The tendon tissue was washed thoroughly in sterile PBS, sliced longitudinally and, for enzyme analysis, separated into tissue from central and peripheral zones as described in chapter 2. Tissue was suspended at approximately 1 g/ml in Krebs-Ringer bicarbonate buffer (2·5 mM Na_2HPO_4, 5 mM $NaHCO_3$, 140 mM NaCl, 4·7 mM KCl, 1·25 mM $MgSO_4$, 2·5 mM $CaCl_2$), pH 7·4, supplemented with 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid), 1% (w/v) BSA (bovine serum albumin) and 11 mM glucose, equilibrated for 5 min. with 95% O_2 : 5% CO_2. Collagenase was added to 0·1% (w/v) and digestion carried out at 37°C. The usual concentration of collagenase for cell isolation is within the range 0·01% - 0·1%; a higher concentration is likely to cause cell damage (Elliot, 1979). Similarly, digestion times of greater than 120 min. were not considered due to the risk of cell damage. Aliquots were taken from the digestion mixture at time periods of 0 - 120 min., cells sedimented by centrifugation at 1600g for 1 min. and cell numbers quantified by deoxyribonucleic acid (DNA) assay by a modification of the method of Kim *et al.* (1988) (see chapter 2). Cell viability was evaluated using 0·2% trypan blue. These experiments indicated that a time of 90 minutes was optimal for digestion (see fig. 4.2).

In subsequent experiments the mixture was filtered through nylon gauze after 90 min. of digestion and washed through with Krebs-Ringer bicarbonate buffer. Cells were sedimented by centrifugation (1600g, 1 min.) and washed once in Krebs-Ringer bicarbonate buffer.

Enzyme extraction

Tendons for this part of the study were collected from nine horses (see appendix 2) and placed into one of three groups; a young group of horses ranging in age from 1 - 3 years (mean age 2 yr), an old group with age range 8 - 14 years (mean

age 10 yr) and a group of tendons with a reddish central core indicating degenerative change (age range 3 - 15 years, mean age 9 yr). Each group consisted of tendons from three horses. Tissue was separated into central and peripheral zones prior to collagenase digestion. Following isolation and washing, the cells were pelleted by centrifugation and stored at -70°C for not more than one month prior to enzyme assays.

Cells were resuspended in 100 - 200 µl of 0·1 M KH_2PO_4 buffer, pH 7·2 (to give approx. 7·5 µg DNA/100 µl), containing 50 µl/ml rat serum, 1 mM DTT (dithiothreitol), 1 mM ADP (adenosine diphosphate) and 0·4% Triton X-100 (iso-octylphenoxypolyethoxyethanol). Cells were frozen and thawed three times, vortex mixing thoroughly between each cycle to ensure rupture of both plasma and mitochondrial membranes. The nuclear pellet was then sedimented at 1000g for 5 min. and stored (-70°C) for DNA assay. DNA was assayed by the method described in chapter 2 following papain digestion for 1 hr at 60°C. The supernatant was kept on ice whilst enzyme assays were carried out.

Lactate dehydrogenase assay

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \overset{\text{LDH}}{\rightleftharpoons} \text{lactate} + \text{NAD}^+$$

Assays of lactate dehydrogenase (LDH) activity were carried out in 1 ml of 50 mM MOPS (3-(N-morpholino) propane-sulphonic acid) /KOH buffer, pH 7·2 plus 1 mM DTT, 2 mM $MgCl_2$, 0.2 mM EGTA (ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N',-tetraacetic acid) and 1 µg/ml rotenone (Rutter, 1988). NADH (nicotinamide adenine dinucleotide) was added to 0·1 mM along with 10 ul of cell extract. The reaction was started by the addition of pyruvate (1 mM). The disappearance of NADH was followed spectrophotometrically at 340 nm and 30°C with a Pye - Unicam PU 8800 spectrophotometer.

Citrate synthetase assay

$$\text{oxaloacetate} + \text{acetyl CoA} + \text{H}_2\text{0} \xrightarrow{\text{CtS}} \text{citrate} + \text{CoA} + \text{H}^+$$

Citrate synthetase (CtS) was assayed by the method of Coore *et al.* (1971). 0.1M Tris (hydroxymethyl) aminomethane·HCl, pH 7·4 was used with the following additions; acetyl CoA (75 µM), DTNB (5,5'-dithio-bis (2-nitrobenzoic acid), 40 µg/ml) and 10 µl of cell extract. The CtS reaction was started by the addition of oxaloacetate

(13 μg/ml) and the increase in absorbance of DTNB followed spectrophotometrically at 412 nm and 30°C. Rates were corrected for any blank rate in the absence of oxaloacetate.

Malate dehydrogenase assay

$$\text{oxaloacetate} + \text{NADH} + \text{H}^+ \overset{\text{MDH}}{\rightleftharpoons} \text{malate} + \text{NAD}^+$$

Malate dehydrogenase (MDH) was assayed as for LDH except that the reaction was started by the addition of oxaloacetate to 0·1 mM (Rutter, 1988).

Glutamate dehydrogenase assay

$$\text{2-oxoglutarate} + \text{NADH} + \text{H}^+ + \text{NH}_4^+ \overset{\text{GDH}}{\rightleftharpoons} \text{glutamate} + \text{NAD}^+ + \text{H}_2\text{O}$$

Glutamate dehydrogenase (GDH) assays were carried out in the basic MOPS/KOH buffer as for LDH (see above), supplemented with 1 mM ADP, 5 mM 2-oxoglutarate and 0·1 mM NADH (Thomas & Denton, 1986). 100 μl of cell extract was used and the reaction started by the addition of NH_4^+acetate (80 mM). The disappearance of NADH was followed as above.

Enzyme activities were expressed as U/mg DNA. One unit (U) is the amount of enzyme that reduces 1 μmol NADH / min. at 30°C or in the case of CtS, the amount of enzyme that produces 1 μmol DTNB-CoA / min. at 30°C.

Glucose utilisation and oxidation

Freshly isolated cells were obtained from three horses (see appendix 2) (mean age 14 yr) by the method described above. As no differences were observed in the levels of CtS or LDH activities between central and peripheral zones, tissue was not divided but left whole to increase cell yield for this part of the work. Cells were resuspended in 210 μl Krebs-Ringer bicarbonate buffer, pH 7·4, supplemented as for digestion (see above) but with 1 mM glucose.

Glucose utilisation was measured by incubation of 100 μl of cell suspension (approx. 0·5 x 10^6 cells), with 1 mM D-[5-^{3}H] glucose (4 μCi/μmol glucose) in tubes

(6 mm x 30 mm) placed inside stoppered scintillation vials. Incubation was carried out for 2 hr at 37°C. The outer vials contained 0·5 ml of water. After incubation metabolism was stopped by the injection of 100 µl of 5% trichloroacetic acid into the tube containing the cells. The vessels were then incubated for a further 24 hr to allow the [^{3}H] water in the incubation tube to equilibrate with the water in the outer vial. Radioactivity in the water was then measured by liquid scintillation counting (Ashcroft *et al.*, 1972).

Glucose oxidation was measured by incubation of 100 µl of cell suspension with 1 mM D-[6-^{14}C] glucose (4 Ci/mol.), except that the outer vials contained 0·5 ml phenylethylamine to trap $^{14}CO_2$. Control samples containing no cells were included and the labelled glucose alone also counted (Ashcroft *et al.*, 1972). These blank values were subtracted and the fluxes of glucose calculated after determination of the specific activity of the radiolabelled substrate.

Statistical analysis

Statistical significance was evaluated using an ANOVA test where horse was a random effect and zone (central or peripheral) and group (young, old or degenerated) were fixed effects. The level of significance was taken as $p \leq 0{\cdot}05$. Data are presented as mean ± S.E.M.

4.3 Results

Isolation of viable tendon cells

Freshly isolated cells, obtained by collagenase digestion of mature equine SDFT, are shown in fig. 4.1. The number of cells collected by collagenase digestion increased until 90 min. of incubation, after this time point cell release slowed down (fig. 4.2). At 90 min. cells were approximately 80% viable as judged by the exclusion of trypan blue (data from 3 separate experiments). Cells were examined under a light microscope and appeared round with a diameter between 5 - 10 μm. A proportion of the cells were single whilst many formed clusters. The number of cells released per gram of tissue was very low, around 0·3% of the total, calculated from cellularity figures (DNA content) obtained in the previous chapters. This is likely to be due to the indigestible nature of the tissue, such that only cells from the cut surfaces are released.

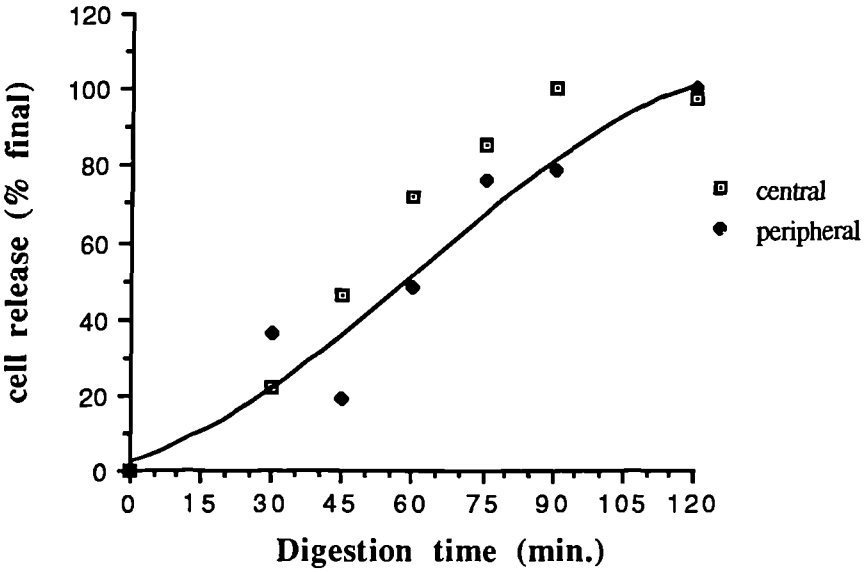
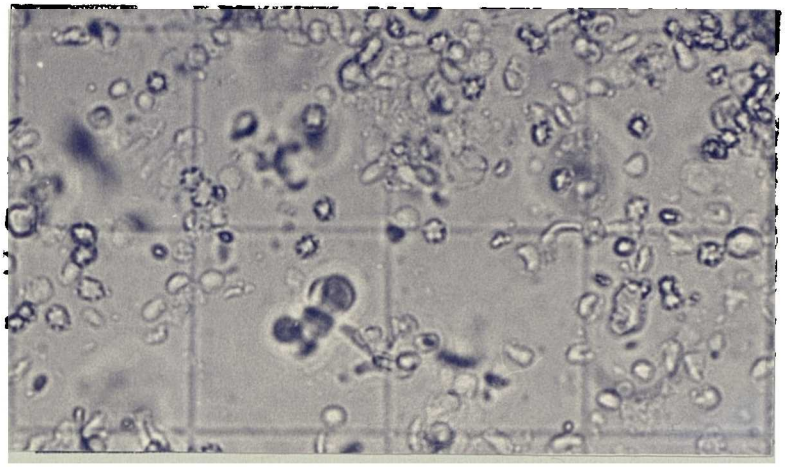


Fig. 4.2 Percentage of total cells released against time of collagenase digestion for tissue from the central and peripheral zone of SDFT. The figure shows data from digestion of tendon tissue obtained from a 15 yr old Thoroughbred horse.



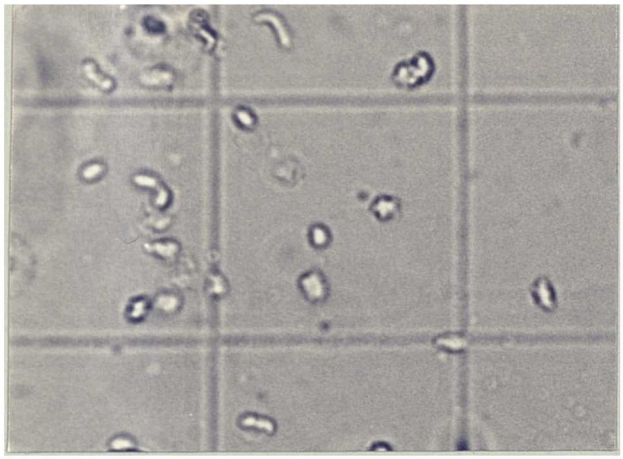


Fig. 4.1 Tenocytes isolated from equine SDFT by collagenase digestion. (1 square = 50 μm)

Enzyme activities

As shown in table 4.1, freshly isolated cells from either zone of the tendon displayed no significant differences in the levels of any of the three measured enzyme activities in the young (≤ 3 yr), old (≥ 8 yr) or degenerated group, suggesting that there is no loss of potential for oxidative metabolism through ageing or with degeneration. There were no significant differences in LDH activities or CtS activities between central and peripheral zones in any of the groups. The peripheral zone of the tendon however had significantly ($p < 0{\cdot}01$) higher MDH activities than the central zone (fig. 4.3) in all three groups. GDH assays were performed on four of the samples and a mean of 0·081 ± 0·007 U/mg DNA was obtained.

Table 4.1 Key metabolic enzyme activities in cells freshly isolated from equine SDFT. Data are presented as mean ± S.E.M. (n = 3). * denotes a significant difference relative to the peripheral tissue.

		Enzyme activity (U/mg DNA)		
Enzyme	Tendon zone	young	old	degenerated
citrate synthetase	central	1·31 ± 0·35	1·47 ± 0·35	1·51 ± 0·35
	peripheral	2·38 ± 0·35	1·43 ± 0·35	1·16 ± 0·35
malate dehydrogenase	central	6·87 ± 1·95 *	7·78 ± 1·95 *	7·58 ± 1·95 *
	peripheral	16·99 ± 1·95	14·55 ± 1·95	10·66 ± 1·95
Lactate dehydrogenase	central	22·68 ± 4·49	20·09 ± 4·49	15·27 ± 4·49
	peripheral	25·74 ± 4·49	14·67 ± 4·49	16·62 ± 4·49

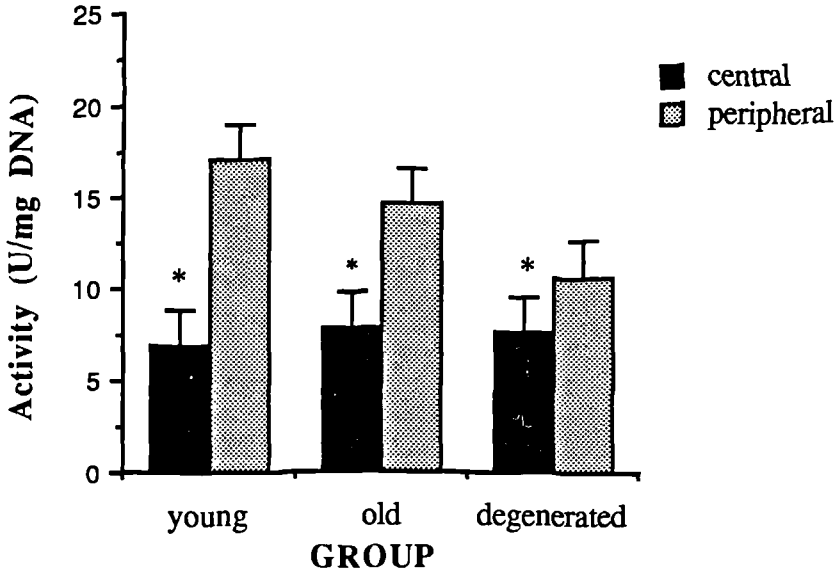


Fig 4.3 MDH activity in central and peripheral zones of young, old and degenerated equine SDFT. Data are given as mean ± S.E.M. (n = 3). * denotes a significant difference relative to the peripheral tissue.

Glucose metabolism.

Glucose utilisation was calculated by measuring the tritiated water given off by the cells when incubated with D-[5-^{3}H] glucose. This measures flux through glycolysis, with ^{3}H label being transfered to H_2O in the reaction catalysed by enolase. Glucose oxidation was measured by the $^{14}CO_2$ given off when cells were incubated with D-[6-^{14}C] glucose. The proportion of utilised glucose (i.e., that passing through the glycolytic pathway at least as far as pyruvate) which is then oxidised by mitochondria (producing CO_2) is thus directly obtained.

Results are expressed as pmoles glucose/100µl cell suspension/hr (table 4.2). The mean ratio of glucose oxidised/ glucose utilised was 0·14 ± 0·03 (data from three animals).

Table 4.2 Glucose utilisation and oxidation by cells isolated from equine SDFT. Data are from separate experiments on cells isolated from three different horses.

	pmoles glucose/100µl digest/hr		
Tendon	1	2	3
Glucose utilization	80·7	170·6	31·3
Glucose oxidation	8·9	32·8	3·7
ratio gluc. oxid./util.	0·11	0·19	0·12

Discussion

Cell isolation

The cell isolation technique produced viable tendon cells from mature SDFT tissue. The low number of cells obtained from the tissue is not surprising given the indigestible nature of the extracellular matrix surrounding the cells. The majority of the tissue remained undigested by collagenase and it seems likely therefore that only cells from the cut surfaces were released. In an attempt to improve cell yield, homogenisation of the tissue prior to collagenase digestion was tried to increase the surface area for enzymatic degradation. This proved unsuccessful however as the collagen fibres simply wrapped around the cutting blade.

The predominant cell type found in tendon tissue are fibroblasts (Holmes, 1971) and those cells released following collagenase digestion were of the characteristic size and shape for such cells. Cells from any remaining loose connective tissue surrounding the tendon might be a source of contamination in the peripheral cell preparation. However, cells from both the central and peripheral zones were of a similar appearance and gave similar enzymological profiles, which suggests that cells from the peripheral tendon were not contaminated with other loose connective tissue cells. Red blood cells are another possible source of contamination of this preparation. However, red blood cells, although visibly present at a low level, have no nucleus or mitochondria and so do not contribute to cell number or oxidative energy metabolism.

The use of freshly isolated cells enables energy metabolism to be studied in cells which have the same complement of enzymes as they would *in vivo*. There are also other reasons for obtaining intact viable isolated cells. Many other studies such as single cell experiments and hormone - receptor binding studies can be carried out on such cells.

Energy metabolism

The results of the enzyme assays have shown that mature equine tenocytes possess Krebs cycle enzymes and therefore mitochondria. The presence of mitochondria indicate that these cells have the capacity for oxidative metabolism. Indeed, the levels of CtS in freshly isolated tenocytes are closely comparable to those for a variety of other mammalian cell types such as fat cells (Martin & Denton, 1970) and lung fibroblasts (Board *et al.*, 1990). This would appear to confirm and extend the view that these cells are fully competent for oxidative metabolism. In further support of this view, the ability of intact equine tendon cells to metabolise an oxidative substrate, glucose, at least partially to CO_2 and H_2O was demonstrated directly. Such metabolism might be predicted for cells with vigorous biosynthetic function and indeed a similar proportion of utilised glucose is oxidised in insulin-secreting pancreatic β-cells (~ 2%, Sener & Malaisse, 1991) as was observed for freshly isolated tendon cells (~14%). Nevertheless, whilst it is likely, one cannot take it for granted that this process is linked to the production of ATP. For example, in brown adipose tissue of young and hibernating mammals the oxidation of substrates and phosphorylation of ADP can become un-coupled (Stryer, 1988). Substrate oxidation is this manner is a means of generating heat in this tissue. The direct demonstration that oxidative metabolism is required to maintain ATP levels in tendon-derived cells is described in the next chapter using cultured cells. "Metabolic inertness" (i.e., low turnover of collagen) reported by some workers for mature tendon (Neuberger *et al.*, 1951; Thompson & Ballou, 1956) cannot therefore be attributed to a loss of oxidative capacity of individual tendon cells (although the overall oxidative capacity of the complete tendon in some species may decline upon maturation as a result of a decrease in the total number of cells per unit mass of tendon (Inglemark, 1948a)). Thus, damage to tendon cells, for example that elicited by hypoxia (Józsa *et al.*, 1982), or by the generation of free radicals leading to mitchondrial damage (Schulze-Osthoff *et al.*, 1992), must be considered as a possible pathway leading to tendon tissue degeneration.

Differences in CtS and LDH activities between central and peripheral zone tissue and between the groups of tendons were not significant with the small group size (n = 3) used in this study. If group sizes were increased differences may have been significant, but not large. Measurement of a maximum enzyme activity is a fairly crude

indicator of flux through a cellular metabolic pathway; substrate and product concentrations and the presence of stimulatory or inhibitory factors will have a large influence on the rate of an enzymatic reaction. It was considered, therefore, that the small differences which may exist (within an order of magnitude) would be of no biological significance and thus further samples, to increase group size, were not analysed. Similarly, the differences in MDH activities observed between central and peripheral zone tissue were considered too small to be of any biological significance.

As CtS activities were similar, central core degeneration can not be explained by the absence of "metabolically active" cells in this region, which has been shown to possess similar total numbers of cells (based on the measurement of DNA) as the peripheral region (chapter 2). Nonetheless, it should be noted that other defects in these cells, particularly in the ability of the cells to synthesise collagen and other matrix components in response to stimuli may exist and could be responsible for this susceptibility.

The observation that mature tenocytes are capable of oxidative metabolism is not surprising. Generally, the percentage of the total ATP production that occurs by glycolysis in the cells of higher organisms is very small compared to the amount formed by oxidative phosphorylation (Stryer, 1988). There are however some special cases in which glycolysis supplies most, and in some cases all of a cell's ATP. For example red blood cells have no mitochondria; all their (limited) ATP production occurs by glycolysis. Also certain types of skeletal muscle fibres (type IIB) contain considerable amounts of glycolytic enzymes but have few mitochondria; during intense muscle activity, glycoysis is the major source of ATP. This is an adaptation to produce ATP very rapidly. There is no reason to believe that such an adaptation is necessary in tenocytes as during exercise there is no demand for rapid cell activity. Thus, tenocytes have the capacity to produce ATP more efficiently allbeit at a slower rate.

Most, if not all, cells are able to withstand very short periods of low oxygen by using anaerobic glycolysis. However, most cells do not have sufficient levels of glycolytic enzymes or sufficient amounts of glycogen to provide the high rates of ATP production by anaerobic glycolysis required to maintain their activities. It seems likely that tenocytes would not be sensitive to acute hypoxia during exercise but are likely to be sensitive to chronic hypoxia. This sensitivity to chronic hypoxia might be increased on training as demand for matrix repair and therefore protein synthesis increases.

In conclusion, mature equine SDFT tenocytes possess mitochondrial enzyme activities and demonstrate the capacity for oxidative metabolism. Hypoxia, therefore, through its effect on energy metabolism represents a possible cause of cellular damage.

Chapter Five

CULTURE OF EQUINE TENDON FIBROBLASTS AND INVESTIGATION OF ENERGY METABOLISM IN THESE CELLS

5.1 Introduction

Degeneration within the central core of the equine superficial digital flexor tendon (SDFT) results from a change in cell function (chapter 3). Suspensions of cells isolated from tissue, obtained as in the previous chapter, are ideal for studying the biochemical make up of the cell and short term affects of agents. However, to study the effects of different physiological conditions on cellular activities such as proliferation and collagen synthesis it is necessary to maintain cells in a viable state for longer periods of time. Most normal cells are anchorage dependent and, unlike transformed and malignant tumour cell lines, depend upon attachment to a solid substrate for survival (Freshney, 1987). Hence it is not possible to carry out longer term experiments on suspensions of freshly isolated cells.

Tissue culture was first devised at the beginning of the century (Harrison, 1907; Carrel, 1912) and, as the name implies, was elaborated first with undisaggregated fragments of tissue with growth restricted to the migration of cells from the periphery of the tissue fragment. As there is no rapid cell proliferation using this technique each experiment requires fresh explants. Since the 1950's dispersed cell cultures have been used either cultured as an adherent monolayer on a solid substrate or as a suspension in the culture medium. Because cell proliferation is often found in such cultures, propagation of cell lines becomes feasible and the technique of cell culture was developed.

There are several major advantages of working with cells grown in culture. The main advantage is that this provides a supply of a homogeneous population of cells growing in a constant environment. This reduces the variation between experimental repeats. Cell culture also enables longer term experiments to be carried out under varied but controlled, conditions. Finally, a major advantage of cell culture when working with tenocytes is that it increases the available number of cells allowing biochemical characterisation. Thus, due to the above advantages and the difficulties in obtaining such cells by collagenase digestion it was decided to grow equine tendon fibroblasts in culture.

In this chapter the energy metabolism characteristics of cultured equine tendon fibroblasts were determined and compared with cells isolated by collagenase digestion of equine SDFT in the previous chapter. If aerobic capacity is similar in cultured and freshly isolated cells the effects of hypoxia on energy metabolism and subsequently cell function can be studied in a culture system. The methods developed for growing and maintaining equine tendon fibroblasts in culture will be used in subsequent chapters for studies of cell survival, growth rates and collagen synthesis.

Hypothesis

Equine tendon fibroblasts can be cultured from explants of SDFT tissue and have a similar aerobic capacity to tenocytes *in vivo* making them suitable for studying cell behaviour under conditions of metabolic stress.

Objectives

1. To grow a stock of equine tendon fibroblasts in culture which could be used for further experiments.

2. To characterise energy metabolism in cultured equine tendon fibroblasts and compare to tenocytes collagenase digested out of whole tendon.

Experimental design

As in the previous chapter, to assess the potential for oxidative metabolism, the maximal activities of key enzymes involved in glycolysis (lactate dehydrogenase) and mitochondrial respiration (citrate synthetase) were measured. In addition, the activities of other mitochondrial enzymes (malate dehydrogenase, oxo-glutarate dehydrogenase, NAD^+ linked isocitrate dehydrogenase and glutamate dehydrogenase) were measured to provide further evidence for the existence of mitochondria. The flux of glucose through glycolysis and the Krebs cycle was assessed directly, in intact cells by radioisotopic methods. The contribution of aerobic metabolism to the maintenance of cellular adenosine triphosphate (ATP) levels was assessed by incubation with a respiratory un-coupler, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). Under normal conditions, the oxidation of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) are coupled by a proton gradient across the

inner mitochondrial membrane to the phoshorylation of adenosine diphosphate (ADP). In the presence of FCCP transfer of electrons from NADH and $FADH_2$ to O_2 proceeds but this is not coupled to the production of ATP. Thus, the cell relies on glycolysis to produce ATP and the contribution of aerobic metabolism to the maintenance of cellular ATP levels can be calculated. A FCCP concentration of 5 μM is sufficient to completely un-couple oxidative phosphorylation (Heytler & Prichard, 1962) therefore this concentration was used in these experiments.

5.2 Methods

Cell culture

Tendon tissue was obtained from half- to full-Thoroughbred horses ranging in age from 6 - 20 years (see appendix 2), slaughtered for reasons other than tendon injury. Lengths of tissue (approximately 1 cm) were taken from the mid-metacarpal region of the SDFT, loose connective tissue was dissected away and the tissue was washed thoroughly several times in sterile PBS (phosphate buffered saline). Tissue was transported on ice in sterile PBS to the tissue culture laboratory. Outer layers of tissue were cut away and the tissue washed in sterile PBS followed by three rinses in sterile water and one in sterile PBS plus antibiotics (streptomycin, 100 μg/ml; benzylpenicillin, 200 U/ml; neomycin, 50 U/ml) and fungizone (amphotericin, 5 μg/ml) at room temperature, before transfer to clean tubes. Washing was continued with three rinses in HBSS (Hank's balanced salt solution) buffered with 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) and completed with sterile PBS plus fungizone and antibiotics. Subsequent steps were carried out in a Laminar flow hood.

Explants of tissue were set up in culture by a modification of the method of Chard *et al.* (1987). Fragments of approximately 1 mm^3 were cut and placed in tissue culture grade plastic petri dishes (60 mm diameter) at five per dish. These were covered with a circular sterile glass cover slip held in place with a spot of sterile grease. DMEM (Dulbecco's modified Eagle's medium) (5 ml) supplemented with 20 mM HEPES, 10% FCS (foetal calf serum) plus antibiotics and fungizone (as above) was added to each dish and the cultures then incubated in a humidified incubator at 5% CO_2 and 37°C. Medium were changed weekly.

The explant derived cells took about one week to appear from the tissue fragment and the about another week to become confluent and cover the coverslip.

Once this had occurred, coverslips were inverted and the medium and explants removed. Cells were then released with 0·05% trypsin in sterile PBS containing 0·02% EDTA (ethylenediamine tetra-acetic acid) and phenol red (0·01%) and transferred to 25cm^2 flasks. When cells reached confluency they were transferred to successively larger flasks and finally maintained in monolayer in 175cm^2 flasks in DMEM supplemented as above. Cells were grown in this way from both central and peripheral zones of the tendon.

Enzyme extraction

Enzyme activities of six different cell lines were measured. These were cells cultured from the central and peripheral zone tissue from three different horses (ages 8, 9 and 15 yr).

Confluent cells (50 000 cells/cm^2) between passages 3 and 5 were trypsin released from 175 cm^2 flasks. Cells were washed twice in 1 ml of ice cold 0.1 M KH_2PO_4, pH 7.2, 50 µl/ml rat serum plus 1 mM DTT (dithiothreitol). After washing cells were resuspended in 200 µl of the above buffer plus 1 mM ADP and 0.4% NP-40 (ethylphenolpoly(ethyleneglycolether)$_n$). Cells were frozen and thawed three times, vortex mixing thoroughly between each cycle to ensure cell rupture and release of enzymes from cytosolic and mitochondrial compartments. The nuclear pellet was then sedimented at 1,000g for 5 min. and stored (-70°C) for deoxyribonucleic acid (DNA) assay. The supernatant was kept on ice whilst enzyme assays were carried out. DNA was assayed by a modification of the method of Kim *et al.*, 1988 (see chapter 2) following papain digestion for 1 hr at 60°C.

Enzyme assays

The assay of lactate dehydrogenase (LDH), malate dehydrogenase (MDH), glutamate dehydrogenase (GDH) and citrate synthetase (CtS) activities were carried out in exactly the same way as for collagenase digested cells (see previous chapter). In addition assays of 2-oxoglutarate dehydrogenase (OGDH) and NAD^+ linked isocitrate dehydrogenase (ICDH-NAD^+), both mitochondrial enzymes involved in the citric acid cycle (see fig. 1.21), were carried out as described below.

2-Oxoglutarate dehydrogenase

$$\text{2-oxoglutarate} + \text{CoA} + \text{NAD}^+ \xrightarrow{\text{OGDH}} \text{succinyl CoA} + CO_2 + \text{NADH}$$

Assay of OGDH activities were carried out in 1 ml of a 50 mM MOPS (3-(N-morpholino) propane-sulphonic acid)/KOH buffer, pH 7·2, plus 1 mM DTT, 2 mM $MgCl_2$, 0·2 mM EGTA (ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N',-tetraacetic acid) and 1 µg/ml rotenone. The following additions were made; NAD^+ (2 mM), TPP (thyamine pyrophosphate, 1 mM), CoA (coenzyme A, 0·1 mM), ADP (1 mM) along with 30 µl of cell extract. The reaction was started by the addition of 2-oxoglutarate (5 mM). The appearance of NADH was followed spectrophotometrically at 340 nm and 30°C with a Pye - Unicam PU 8800 spectrophotometer (McCormack & Denton, 1979).

Isocitrate dehydrogenase

$$\text{D,L-isocitrate} + \text{NAD}^+ \rightleftharpoons^{\text{ICDH}} \text{2-oxoglutarate} + CO_2 + \text{NADH} + H^+$$

ICDH-NAD^+ assays were carried out in the basic MOPS/KOH buffer as for OGDH (see above). 2 mM NAD^+ and 1 mM ADP were added along with 30 µl of cell extract. The reaction was started by the addition of D,L-isocitrate (5 mM) and the appearance of NADH followed spectrophotometrically at 30°C and 340 nm (Denton *et al.*, 1978).

Glucose utilisation and oxidation

Six different cell lines, obtained from four different horses were used for this part of the work. Confluent cells between passages 3 and 5 were used. Cells were trypsin released and washed in Krebs-Ringer bicarbonate buffer (2·5 mM Na_2HPO_4, 5 mM $NaHCO_3$, 140 mM NaCl, 4·7 mM KCl, 1·25 mM $MgSO_4$, 2·5 mM $CaCl_2$), pH 7·4, supplemented with 10 mM HEPES, 1% (w/v) BSA (bovine serum albumin) and 1 mM glucose gassed for 5 min. with 95% CO_2 : 5% O_2 (as used for glucose utilisation experiments on collagenase isolated cells). Cells were resuspended (approx. 1·5 x 10^6 cells/ml) in the same buffer.

Glucose utilisation and oxidation were measured in the same way as for collagenase isolated cells (Ashcroft *et al.*, 1972; see chapter 4). 100 µl of cell suspension was incubated with [5-^{3}H] glucose (4 µCi/µmol glucose) at 37°C for 1 hr.

[^{3}H] water trapped in the outer vial was then measured by liquid scintillation counting. Glucose oxidation was measured by incubation of 100 µl of cell suspension with [6-^{14}C] glucose (4 µCi/µmol. glucose) and trapped $^{14}CO_2$ measured. All incubations were carried out in duplicate. Control experiments containing no cells were included and the labelled glucose alone also counted.

The remaining cell suspension after the removal of 100 µl aliquots for the experiments was then used for a DNA assay. Cells were sedimented by centrifugation at 1600g for 2 min. and stored at -70°C prior to assay. DNA was assayed by a modification of that of Kim *et al.* (1988) (see chapter 2) following papain digestion for 1 hr at 60°C.

Incubation of cells in the presence of FCCP

Confluent cultured cells were trypsin released, washed in buffer and resuspended in Krebs-Ringer bicarbonate buffer (see above) supplemented with 10 mM HEPES, 1% BSA and 11 mM glucose (500 000 cells/ml). Six incubations were set up (in duplicate) with 1 ml of cell suspension without FCCP present. A further six vessels were set up (in duplicate) with 1 ml of cell suspension plus 5 µM FCCP and incubated at 37°C in a water bath for times of 30, 40, 50, 60, 75 and 90 minutes. After the appropriate time the reaction was stopped by the addition 15 µl of 70% perchloric acid and frozen immediately on dry ice. At the end of the experiment samples were thawed and neutralised with 5 M KOH, 100 mM HEPES and 2 mM EDTA (ethylenediamine tetra-acetic acid). Sediment was removed by centrifugation before the assay of ATP.

ATP assays

ATP assays were carried out essentially as done by Stanley & Williams (1969) using firefly luciferase and single photons detected with a photomultiplier in a modified liquid scintillation counter.

$$\text{ATP} + \text{luciferin} \longrightarrow \text{luciferyladenylate} + O_2 \xrightarrow{\text{luciferase}} \text{oxyluciferin} + \text{light}$$

Reaction buffer; sodium arsenate (38 mM), sodium phosphate (7·7 mM) and $MgSO_4$ (16·8 mM) was prepared immediately before use. Reaction buffer (2 ml) was placed in plastic vials, 50 µl of cell suspension was added to each vial; blanks were prepared by adding 50 µl of cell suspension alone. The reaction was started by the

addition of 10 µl of luciferase and counting was performed over 30 s. Levels of ATP in the experimental samples were calculated from a standard curve (10 - 500 pmol ATP).

Statistical analysis

Statistical significance was evaluated using an ANOVA test where horse was a random effect and zone (central or peripheral) was a fixed effect. The level of significance was taken as $p \leq 0{\cdot}05$. Data are presented as mean ± S.E.M.

5.3 Results

Cell culture

Equine tendon fibroblasts were successfully grown from the SDFT of 10 different horses ranging in age from 6 to 20 years. In all cases, after about one week cells could be seen growing from the periphery of the explant of tissue. After the second week, coverslips were completely covered with confluent cells. At this time tissue explants were removed and cells sub-cultured into tissue culture flasks. Cells had the characteristic appearance of fibroblasts; cell processes protruded in a star-like shape in sparse cell cultures (fig. 5.1) and as confluency was reached cells became spindle shaped producing a parallel array (fig. 5.2). The cultured fibroblasts were capable of synthesis and secretion of collagen (see chapters 6 &7). Cells reached confluency (50 000 cells/cm^2) every three or four days following sub-culture until around passage 14 at which point cell growth began to decline.

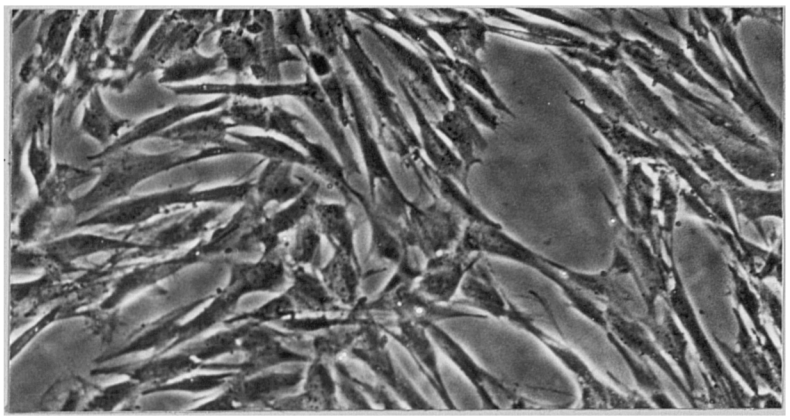


Fig. 5.1 Sparse culture of equine SDFT explant-derived fibroblasts. (x350)

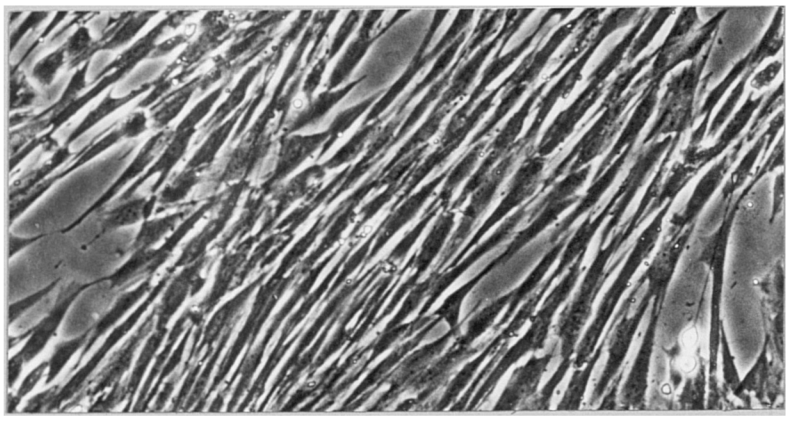


Fig. 5.2 Confluent culture of equine SDFT explant-derived fibroblasts. (x350)

Enzyme activities

Table 5.1 Key metabolic enzyme activities in cells cultured from the central and peripheral zone of equine SDFT. Data are presented as mean ± S.E.M. (n = 3).

	Enzyme activity (U/mg DNA)	
Enzyme	central zone	peripheral zone
Lactate dehydrogenase	215 ± 32	229 ± 32
Citrate Synthetase	1·63 ± 0·52	2·14 ± 0·52
Malate dehydrogenase	37·5 ± 2·0	43·1 ± 2·0
2-oxo-glutarate dehydrogenase	0·16 ± 0·04	0·17 ± 0·04
Isocitrate dehydrogenase	0·04 ± 0·02	0·05 ± 0·02
Glutamate dehydrogenase	0·20 ± 0·09	0·21 ± 0·09

Both anaerobic and aerobic enzyme activities were present in all cell lines measured. Enzyme activities did not differ significantly between cells cultured from the peripheral or central zone tissue of the tendon.

A comparison of enzyme activities between cultured cells and freshly isolated cells (using values obtained for the old group of horses as they were the same age range as the horses used to obtain cultured cells) was made. LDH activities increased significantly ($p = 0·001$) by approximately ten fold in cultured cells from both the central region (215 ± 32) and the peripheral region (229 ± 32),compared to collagenase isolated cells (central, 20·09 ± 4·49; Peripheral, 14·67 ± 4·49).(fig. 5.3). MDH activities were also significantly higher ($p < 0·000$) in the cultured cells (central, 37·5 ± 2·0; Peripheral, 43·1 ± 2·0) compared to collagenase isolated cells (central, 7·78 ± 1·95; Peripheral, 14·55 ± 1·95) (fig. 5.4). However, there were no differences in CtS activity between the two cell sources from either region (fig. 5.5) (cultured cells: central, 1·63 ± 0·52; peripheral, 2·14 ± 0·52; isolated cells: central, 1·47 ± 0·35; peripheral, 1·43 ± 0·35).

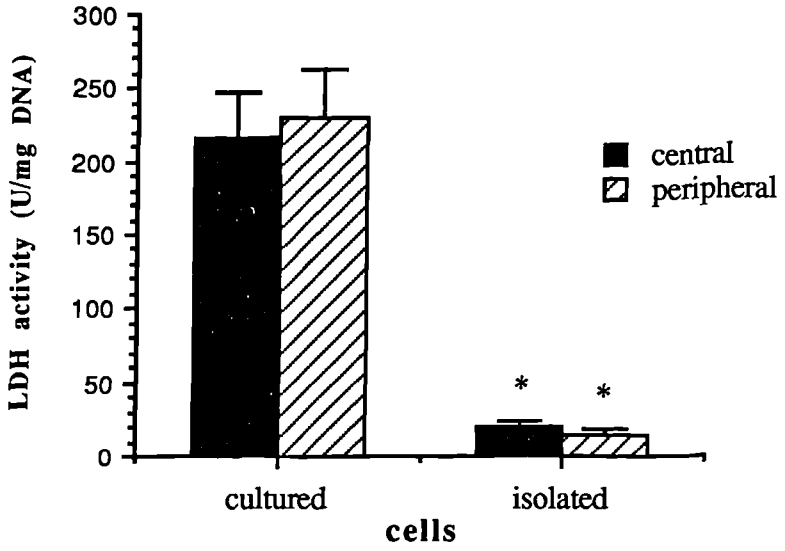


Fig. 5.3 LDH activities of cultured cells and collagenase isolated cells from central and peripheral zones of equine SDFT. (n = 3) Cells from both sources were obtained from horses in the age range 8 - 15 yr. * denotes a significant difference relative to the cultured cells.

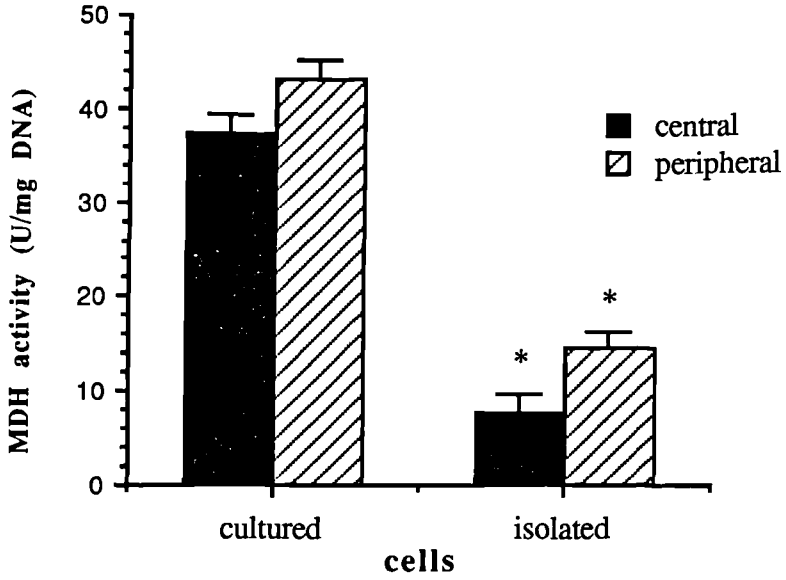


Fig. 5.4 MDH activities of cultured cells and collagenase isolated cells from central and peripheral zones of equine SDFT. (n = 3) Cells from both sources were obtained from horses in the age range 8 - 15 yr. * denotes a significant difference relative to the cultured cells.

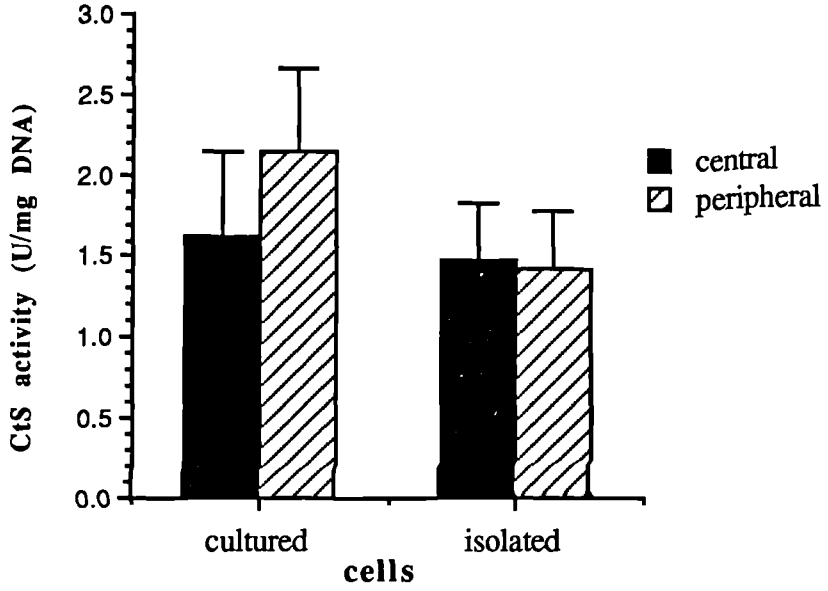


Fig. 5.5 CtS activities of cultured cells and collagenase isolated cells from central and peripheral zones of equine SDFT. (n = 3) Cells from both sources were obtained from horses in the age range 8 - 15 yr.

Metabolism of glucose

The amount of glucose metabolised by cultured equine fibroblasts is given in table 5.2. The proportion of glucose utilised which is subsequently oxidised is also given and does not differ significantly between cells cultured from either the central or peripheral zone tissue. This ratio, however, is considerably lower in cultured cells compared to freshly isolated cells.

Table 5.2 Glucose metabolism by cultured explant-derived tendon fibroblasts. Data are presented as mean ± S.E.M. (n = 3)

fate of glucose	rate of metabolism (pmol/10^3cell/hr) in cultured cells from tendon region:	
	central	peripheral
oxidation of D-[6-^{14}C] glucose	0·97 ± 0·42	1·19 ± 0·42
utilization of D-[5-^{3}H] glucose	161·7 ± 14·3	205·4 ± 14·3
proportion of glucose oxidised (x10^{-3})	6·51 ± 2·53	5·95 ± 2·53

ATP levels in the presence and absence of FCCP

FCCP significantly ($p < 0·001$) reduced ATP levels by about 20% at all time points studied (fig. 5.6). The decline in ATP levels from 30 - 90 min. was not significant.

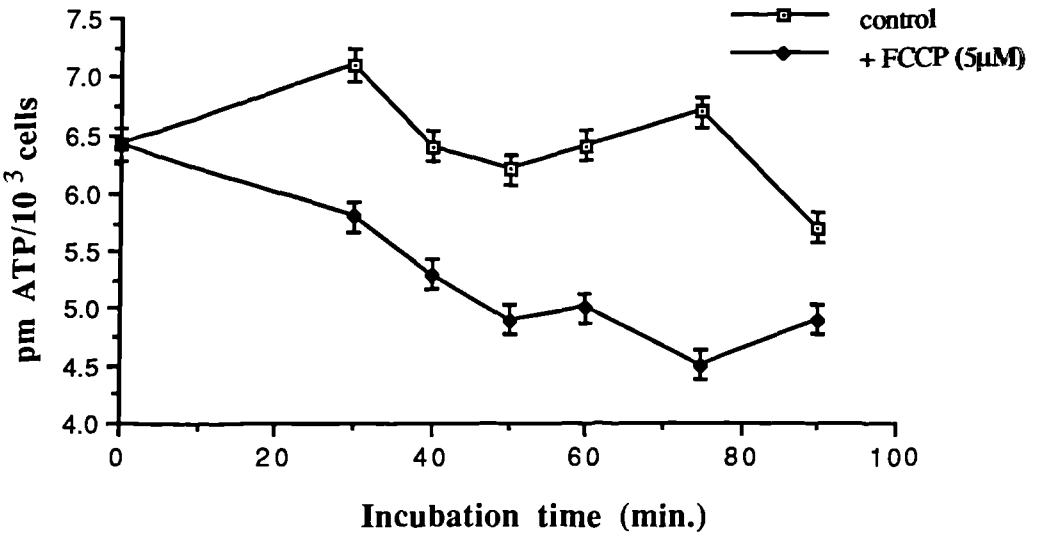


Fig. 5.6 ATP levels within explant-derived cultured SDFT fibroblasts incubated with and without the presence of FCCP.

5.4 Discussion

The technique used for growing cells from explants of tissue provided a convenient method for culturing equine SDFT fibroblasts. Apart from the greater numbers of cells which can be obtained using this approach, another advantage is that this allows the tendon cells to be examined without the risk of contamination with blood or other cell types. The cultured cells obtained in this way would appear to be genuine tendon fibroblasts, and were capable of the synthesis of type I and type III collagen (see chapters 6 & 7).

The levels of oxidative enzymes in these cells were similar to levels in the freshly isolated cells (table 4.1). Consistent with the data obtained with freshly isolated cells, no difference in citrate synthetase levels were found between cells cultured from central and peripheral zones.

In contrast to the similarity in CtS activities for the freshly isolated and cultured cells, LDH levels were markedly higher in the cultured cells. Furthermore, the proportion of the utilised glucose subsequently oxidised was also much lower in the cultured cells, which is consistent with the diversion of glycolytically-derived pyruvate away from mitochondrial (i.e. oxidative) metabolism. This adaptation, which probably involves the induction of the LDH A (muscle type) gene (Li, 1989) may allow rapidly dividing fibroblasts to survive in anoxic conditions as for example during wound healing (Anderson *et al.,* 1989), a situation which is partially mimicked during rapid growth in culture. This shift in metabolic profile of the cultured cells with respect to the freshly isolated cells might be important if this reflects changes which occur in the intact tissue upon regeneration after injury. Nevertheless, the cultured cells appeared to depend at least to some extent upon aerobic metabolism for the maintenance of intracellular ATP levels, since these were significantly lowered by the presence of the respiratory un-coupler, FCCP.

Equine tendon fibroblasts grown during the course of the present work should allow the likely effects on tendon matrix synthesis of such factors as hypoxia, free radicals and hyperthermia to be examined in a convenient *in vitro* system. These experiments have shown that cultured tendon fibroblasts have a similar aerobic capacity to tenocytes *in vivo*, however, increased capacity for, and dependence on, anaerobic metabolism in cultured cells must be considered when interpreting results of metabolic stress studies *in vitro*.

Chapter Six

EFFECT OF HYPOXIA AND FREE RADICALS ON TENDON FIBROBLASTS - AN *IN VITRO* STUDY

6.1 Introduction

Hypoxic cell damage

Degenerative changes in the equine superficial digital flexor tendon (SDFT) are most commonly observed in the mid-metacarpal region of the tendon (Webbon, 1977). This region of the tendon is the least well vascularised (Strömberg, 1971) and it has been suggested that the degenerative changes may be caused by a poor blood supply resulting in tissue hypoxia in the central core of the tendon (Strömberg, 1971; Fackelman, 1973). A similar aetiology has been proposed for human Achilles tendon degeneration (Arner *et al.*, 1959; Burry & Pool, 1973). In chapters 3 & 4 it was shown that mature tenocytes possess mitochondrial enzymes and that oxidative metabolism is necessary to maintain cellular adenosine triphosphate (ATP) levels. Low oxygen tension may therefore, be expected to have a detrimental effect on tenocyte function by compromising their energy metabolism.

Józsa *et al.* (1982) examined degenerated human Achilles tendon tissue and observed numerous "hypoxic" alterations to mitochondria, endoplasmic reticulum, ribosomes, nuclei and the number of lysosomes in tenocytes. The effects of low oxygen tension have been studied *in vitro* using fibroblasts from human Achilles tendon, skin and lung (Webster & Burry, 1982). Cell proliferation, total protein synthesis and collagen synthesis were measured at oxygen tensions of 150 mm Hg (atmospheric partial pressure of oxygen), 75 mm Hg and 20 mm Hg. Tendon cell proliferation was not altered by sub-atmospheric oxygen concentrations. Total protein synthesis however was reduced at 20 mm Hg oxygen pressure and this was most marked in sparse cell cultures. Collagen synthesis was less affected but was significantly reduced at 20 mm Hg in the sparse cell cultures. Fibroblasts from different tissues showed a difference in sensitivity to low oxygen tension, the tendon fibroblasts being the least sensitive. This may reflect an adaptation of tendon cells to enable them to survive low oxygen tensions which may be experienced *in vivo*.

Measurement of oxygen tension within tendon tissue has not been made. In the rabbit ear chamber however, oxygen electrodes have recorded an oxygen tension of 5 mm Hg at a distance of 70 - 80 μm from the nearest capillary (Silver, 1969). The capillaries in tendon run between fibre bundles and a distance of 70 - 80 μm between a tendon cell and blood supply might easily be approached or even exceeded. If this is the case oxygen tensions employed by Webster and Burry (1982) might not have been low

enough to study the true effects of hypoxia on tendon cells. In their experiments a shift towards anaerobic metabolism was not demonstrated. Chronic hypoxia therefore represents a possible cause of tendon cell damage or change in function which may lead to tissue degeneration.

Post-ischaemic cell damage

Whilst molecular oxygen is essential for aerobic life forms, inappropriate metabolism of O_2 can be extremely toxic to the organism. Under normal circumstances molecular oxygen is reduced tetravalently (four electrons are removed from two oxygen atoms in a single process) by the cytochrome system in mitochondria as a normal part of oxidative metabolism (fig. 6.1). Even under normal circumstances, however, a small proportion (approx. 1% - 5%) of the oxygen reduced to water undergoes reduction by a divalent pathway with the consequent production of low levels of free radical intermediates (Bulkley, 1987). These intermediates, including O_2^-, H_2O_2, and $OH\cdot$, are very unstable and hence highly reactive and cytotoxic (McCord, 1983; Bulkley, 1983). Aerobic cells have evolved endogenous control mechanisms that react with the low level of free radicals produced thus terminating the reaction without causing tissue damage (Bulkley, 1987). Superoxide dismutase (SOD) and catalase are two examples of the so called free radical scavengers. Other endogenous defence mechanisms include reduced glutathione, physiologic metal chelators and α-tocopherol.

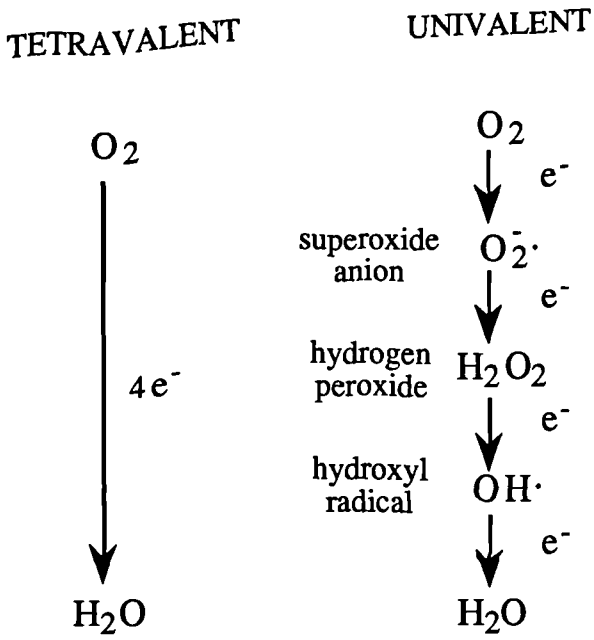


Fig. 6.1 The tetravalent and univalent oxidation of oxygen to water.

The superoxide free radical ($O_2^{-}\cdot$) can also be generated as a by-product of the oxidation of hypoxanthine by the enzyme xanthine oxidase (XO) following tissue ischaemia and reperfusion (fig. 6.2). This situation could occur in the equine SDFT as a result of exercise. During tendon loading, intratendinous blood vessels will be intermittently occluded and perfusion impaired and this may result in tissue ischaemic hypoxia. When loading has ceased normal blood flow returns and generation of free radicals by the action of XO may occur.

The activity of XO is usually low in tissues but following ischaemia increases (Roy & McCord, 1983). This is due in part to the proteolytic conversion of another enzyme, xanthine dehydrogenase, into XO (McCord, 1984). During prolonged ischaemia there is a depletion of high energy phosphate compounds manifested by a decrease in ATP and ADP (adenosine diphosphate) levels in the cell. The adenosine monophosphate (AMP) levels increase and AMP is subsequently catabolised resulting finally in the formation of the free purine base hypoxanthine (Jennings *et al.*, 1981). Because ATP levels decrease, Ca^{2+} can no longer be pumped out of the cytosol by Ca^{2+}-ATPase and there is an influx of Ca^{2+} into the cell. This influx of Ca^{2+} may activate a Ca^{2+} dependant protease in the cytosol (Grisham & McCord, 1986). The protease then attacks the enzyme xanthine dehydrogenase converting it to xanthine oxidase. Xanthine oxidase catalyses the following reaction

$$\text{hypoxanthine} + 2\,O_2 + H_2O \xrightarrow{XO} \text{xanthine} + 2\,O_2^{-}\cdot$$

Ischaemia therefore primes the tissue for reperfusion damage because a new enzyme (XO) has been formed in addition to one of its required substrates (hypoxanthine). On reperfusion of the tissue, oxygen is introduced and there is a burst of XO - generated $O_2^{-}\cdot$ in the tissue with subsequent H_2O_2 and HO· production.

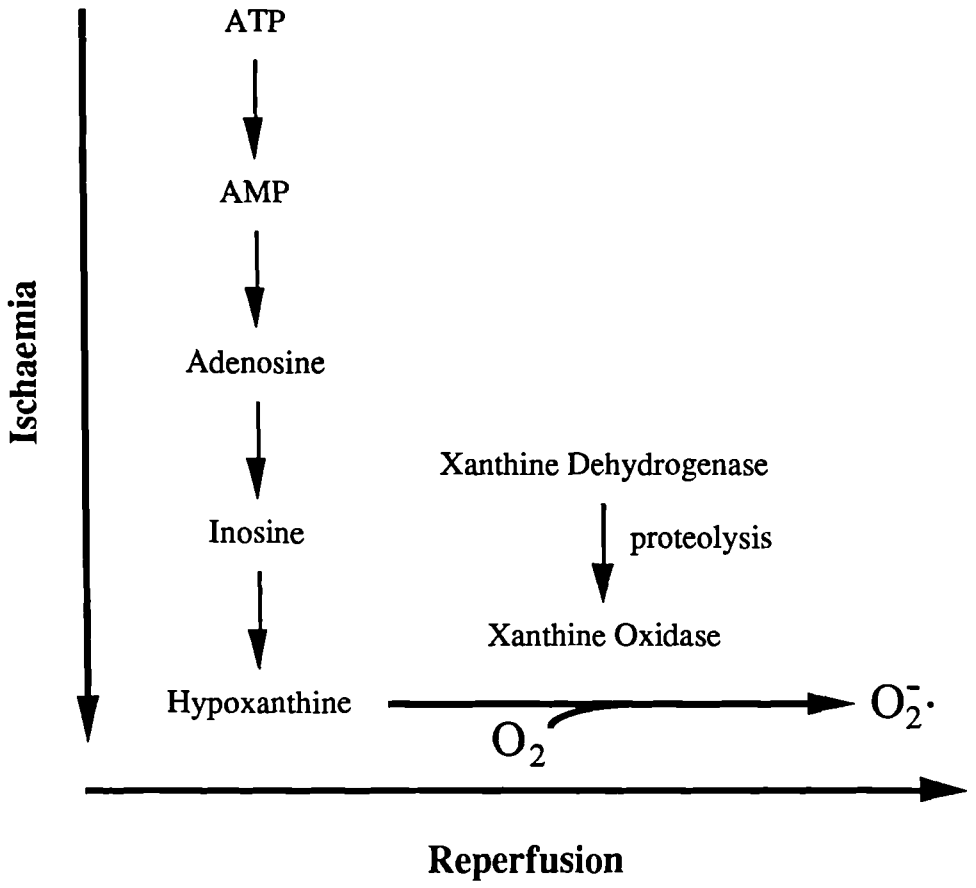


Fig. 6.2 Proposed mechanism for O_2 radical production in ischaemic tissue. (Grisham & McCord, 1986)

Oxygen free radical molecules are unstable due to an un-paired electron in their outer shell and hence, react rapidly with other molecules donating or accepting electrons to stabilise their own configuration. Such a reaction causes profound changes in the conformation of adjacent molecules which is reflected as severe tissue damage. Free radicals can damage deoxyribonucleic acid (DNA), impair DNA synthesis, denature proteins and lead to peroxidation of membrane lipids (Sinclair, 1990). Many of these reactions not only produce direct injury but by donating the extra unpaired electron to those molecules generate secondary radical species that are themselves capable of propagating this injury process. Free radical metabolites of oxygen have been

implicated in ischaemia - reperfusion injury in multiple tissues, including the brain, heart, stomach, small intestine, pancreas, kidney, liver, muscle and skin flaps (Puig *et al.*, 1989).

Reperfusion damage by the generation of oxygen free radicals requires a period of ischaemia followed by reperfusion. Ischaemic hypoxia may occur when the SDFT is loaded during exercise. Furthermore the increased temperatures experienced in the central core of the tendon during exercise (see chapter 7) may potentiate damage caused by a reduction in blood flow by increasing metabolic rate and further running down ATP levels increasing the effect of ischaemia. When exercise has ceased normal blood flow returns and thus generation of free radicals and reperfusion injury may occur. Thus, acute hypoxia may result in tendon cell damage.

Hypothesis

Hypoxia results in a change in tendon cell function or causes cell damage, due to either an increased dependence on anaerobic metabolism for ATP production, or to oxygen free radical generation. This results in altered matrix metabolism, leading to tissue degeneration.

Objectives

1. To measure tendon fibroblast proliferation rates and collagen secretion *in vitro* following prevention of ATP production by aerobic pathways using an un-coupler of oxidative phosphorylation.

2. To determine the effect of different concentrations of free radicals on tendon fibroblast proliferation rates and collagen secretion *in vitro*.

6.2 Methods

Cell culture

Tendon fibroblasts were grown in culture from explants of equine SDFT central zone tissue by the method described in chapter 5. Cultured cells were maintained in monolayer in 175 cm^2 flasks in DMEM (Dulbecco's modified Eagle's medium) supplemented with 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid), 10% FCS (foetal calf serum), antibiotics (streptomycin, 100 µg/ml; benzylpenicillin, 200 U/ml; neomycin, 50 U/ml) and fungizone (Amphotericin, 5 µg/ml). Experiments were carried out on confluent cells between passages 3 and 10.

Growth rates

Cell growth rate was determined by measuring DNA synthesis using [methyl-^{3}H]thymidine incorporation. Trypsinised cells were plated into 24 well plastic plates at a density of 2·5 x 10^4 cells/well in 1 ml of DMEM plus 20 mM HEPES, 10% FCS, antibiotics and fungizone. Growth plates were returned to the 37°C incubator for 24 hr during which time cells were able to establish on the multiwell plates. Medium was then removed and replaced with 1 ml of DMEM supplemented as above, except the FCS concentration was lowered to 1%. After 24 hr the reagent required to produce the experimental condition was added. Twenty four hours later 5 µCi [methyl-^{3}H] thymidine (specific activity 25 Ci/mmol) were added to each well except for blank wells and incubation continued for a further 24 hr. After this time 5 µCi of labelled thymidine was added to the blank wells and the medium immediately decanted from all wells. Cells were washed three times with sterile PBS (phosphate buffered saline) and then lysed with 0·5 ml of 19 M formic acid. The resulting solution was transferred to scintillation vials and 2 ml of scintillant (Packard Ultima Gold) added. Radioactivity in the samples was determined by counting on a scintillation counter (Packard Tri-Carb 1900CA).

Un-coupling of oxidative phosphorylation

The effect of hypoxia was simulated *in vitro* by the addition of a respiratory un-coupler; FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone). In the presence of FCCP oxidation of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) is not coupled to the phosphorylation of ADP and the cell is therefore only able to produce ATP by glycolysis. It was shown in chapter 5 that incubation in the presence of 5 µM FCCP (sufficient to completely un-couple oxidative

phoshorylation, Heytler & Prichard, 1962) significantly reduced ATP levels in explant-derived tendon fibroblasts by about 20%. In the growth rate experiments six different FCCP concentrations of 0·001, 0·01, 0·1, 1, 5, and 10 μM were used. Controls wells with no FCCP present were also set up.

Generation of free radicals

Free radicals were generated in the medium by two different methods (Murrell *et al.*, 1991). In the first, H_2O_2 was added at a range of concentrations (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3}M). Control wells with no H_2O_2 present were also set up. The second method of free radicals generation involved the addition of glyceraldehyde at the following concentrations, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} and 10^{-2}M. Further experiments were carried out with H_2O_2 at the concentrations above plus the free radical scavengers catalase (250 U/ml) and superoxide dismutase (SOD, 20 U/ml), to check that any effect of H_2O_2 on growth rates was due to the generation of free radicals. Free radical scavengers were also added to the control wells in the absence of H_2O_2.

$$2\,H_2O_2 \xrightarrow{\text{catalase}} 2\,H_2O + O_2$$

$$2\,O_2^- + 2\,H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

Each different condition under which growth rates were studied was carried out at least 12 times using three different cell lines. Blank values were subtracted from experimental values and growth rates expressed as a percentage of the controls. Statistical significance was evaluated using an ANOVA test.

Collagen production

Explant-derived tendon fibroblasts were seeded into 25 cm^2 flasks and when cells had become confluent the medium was replaced with 5 ml of DMEM supplemented with 1% FCS, 100 μg/ml β aminopropionitrile and 50 μg/ml ascorbate. Hypoxia was simulated by the addition of FCCP to the medium at a concentration (5 μM) sufficient to reduce cellular ATP levels without causing cell death. The effect of free radicals was studied by inclusion of H_2O_2 at a concentration (10^{-6}M) just less than that which causes cell inhibition (see results of growth rate experiments). Flasks were returned to the 37°C incubator for 36 hr. In experiments where collagen synthesis was followed by the incorporation of proline, 5 μCi of [U-^{14}C]-proline (specific activity

260 Ci/mol) was added after 12 hr and the cells incubated for a further 24 hr. The medium was then decanted, placed on ice and proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 mM ethylmaleimide and 25 mM ethylenediamine tetra-acetic acid) added. Cell number in each flask was determined by DNA assay following papain digestion (chapter 2).

Preparation of collagen for analysis

Protein was precipitated from the decanted medium by the addition of solid ammonium sulphate to 30% saturation, and the solution left at 0°C for 2 hr. Precipitated collagen was sedimented by centrifugation at 40000 g at 4°C, for 30 min. The pellet was resuspended in 0·5 ml of 0·5 M acetic acid, and digested with pepsin (100 μg/ml) for 6 hr at 4°C to remove the non-helical telopeptides. After dialysis overnight against distilled water, samples were lyophilised and resuspended in approximately 200 μl (volume was adjusted to account for a small variation in cell number between each flask) of sodium dodecyl sulphate (SDS) sample buffer (125 mM Tris, pH 6·8, 2% SDS, 10% glycerol, 0·01% bromophenol blue) either with 2% (v/v) β-mercaptoethanol (for the analysis of type III collagen) or without (for type I collagen analysis). Samples were heated at 60°C for 30 min. prior to electrophoresis to dissociate the polypeptide chains.

Analysis of collagen

The pepsin-digested whole collagen α chains were separated by SDS polyacrylamide gel electrophoresis (Laemmli, 1970) on a 7·5% gel using the "Hoffer" design (LKB) gel electrophoresis equipment. Samples (25 μl) were loaded in duplicate (reduced and unreduced) on separate gels and electrophoresis carried out at a constant current of 35 mA/gel generating a voltage of about 100V. Following separation, protein was transferred to Immobilon P membranes overnight using a BioRad transfer system (Trans Blot) at a constant voltage of 60 V and a limiting current of 300 mA. After protein transfer, Immobilon P blots were blocked by washing in Tris buffered saline (TBS) (0·05 M Tris·HCl, pH 8·0, 0·1 M NaCl)/4% milk powder and probed by incubation for 2 hr at room temperature with goat anti-type I collagen (unreduced samples) or goat anti-type III collagen (reduced samples) antibodies diluted 1:1000 in each case with TBS/4% milk powder. Blots were washed in TBS/0·1% polyoxyethylenesorbitan monolaurate (TWEEN) (3 x 5 min.) and blocked in TBS/4% milk powder (10 min.). Blots were then probed with a second alkaline phosphatase labelled rabbit anti-goat IgG (whole molecule) antibody for 2 hr at room temperature.

TBS/0·1% TWEEN washes were repeated and blots developed with alkaline phosphatase substrate (100 μg/ml 5-bromo-4-chloro-3-indolylphosphate, 0·5 μg/ml nitro blue tetrazolium in 0·1 M Tris, pH 9·7, 0·1 M NaCl and 0·1 M $MgCl_2$), allowed to dry and photographed. Radioactivity in separated collagen peptide bands was determined by autoradiography after soaking the blots in "Amplify" (Amersham), drying, and exposure to Kodak diagnostic film (XRP-6) for 7 days at -70 °C. Each condition under which collagen production was studied was repeated 5 times using 3 different cell lines.

Immuno-detection of secreted collagen by the method described above is not quantitative but, by comparison with control cells, increase or decrease in appearance of each collagen type in the medium under different conditions can be determined. Autoradiography will demonstrate whether cells were synthesising new collagen during the course of the experiment.

The antibodies used for the detection of type I and III collagen were commercially available preparations that had been raised against a mixture of human and bovine collagen. Their specificity for equine collagen was checked using type I and type III collagen standards prepared from equine foetal skin by pepsin digestion and salt fractionation. Standard preparations were separated on 7·5% gels in reduced and unreduced forms by the method described above. Protein bands were visualised either by staining with Coomassie brilliant blue or by transfer to Immobilon P membrane and immuno-detection.

6.3 Results

Effect of FCCP on cell growth rate

The presence of FCCP at concentrations of 0·001 M to 0·1 M had no effect on tendon fibroblast growth rates relative to control cells cultured in the absence of FCCP. At concentrations of 1 μM and 5 μM FCCP there was a decrease in the growth rate but this was not significant. At 10 μM FCCP growth rates were significantly ($p < 0·001$) decreased (fig. 6.3). Tendon fibroblast survival rate showed a non-linear dose response over the range of concentrations used with a sharp decline occurring between 5 and 10 μM FCCP.

Effect of free radicals on cell growth rate

The addition of glyceraldehyde to the medium at low concentrations (10^{-7} M - 10^{-5} M) had no significant effect on tendon fibroblast growth rates. At higher concentrations (10^{-4} M - 10^{-2} M) cell growth was completely inhibited. [methyl-^{3}H]-thymidine counts at these higher concentrations were below those of the blank wells indicating that cell death had occurred (fig. 6.4).

Low concentrations of hydrogen peroxide (10^{-8} M - 10^{-6} M) had no effect on the growth rate of tendon fibroblasts. At concentrations of 10^{-5} M H_2O_2 growth was inhibited. Inhibition was significant ($p < 0·001$) at 10^{-4} M H_2O_2 when growth rates were only 10% of the control rate and at a concentration of 10^{-3} M H_2O_2 cell death occurred (fig. 6.5). This represents a non-linear response over the range of concentrations used.

The addition of the free radical scavengers, catalase and SOD, to the medium eliminated the growth inhibition observed in the presence of H_2O_2 at concentrations of 10^{-5} M to 10^{-3} M. In contrast to H_2O_2 alone, at these concentrations the addition of free radical scavengers stimulated growth (fig. 6.6) and this became significant ($p < 0·001$) at 10^{-3} M H_2O_2.

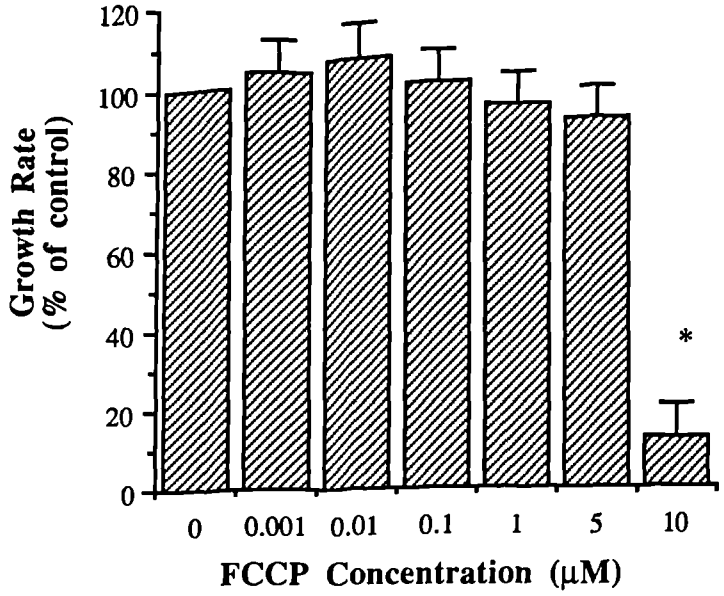


Fig. 6.3 Equine tendon fibroblast growth rates in the presence of FCCP. Data are presented as mean ± S.E.M. n = 3 (each in quadruplicate) Statistical significance was evaluated using ANOVA. L.D.S. = 24·9%, * denotes a significant difference relative to control cells.

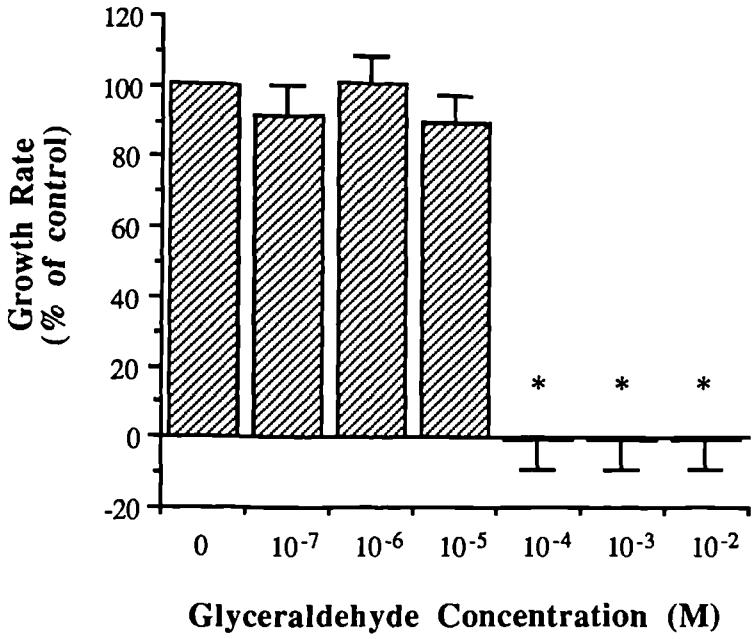


Fig. 6.4 Equine tendon fibroblast growth rates in the presence of glyceraldehyde. Data are presented as mean ± S.E.M. n = 3 (each in quadruplicate) Statistical significance was evaluated using ANOVA. L.D.S. = 24·7%, * denotes a significant difference relative to control cells.

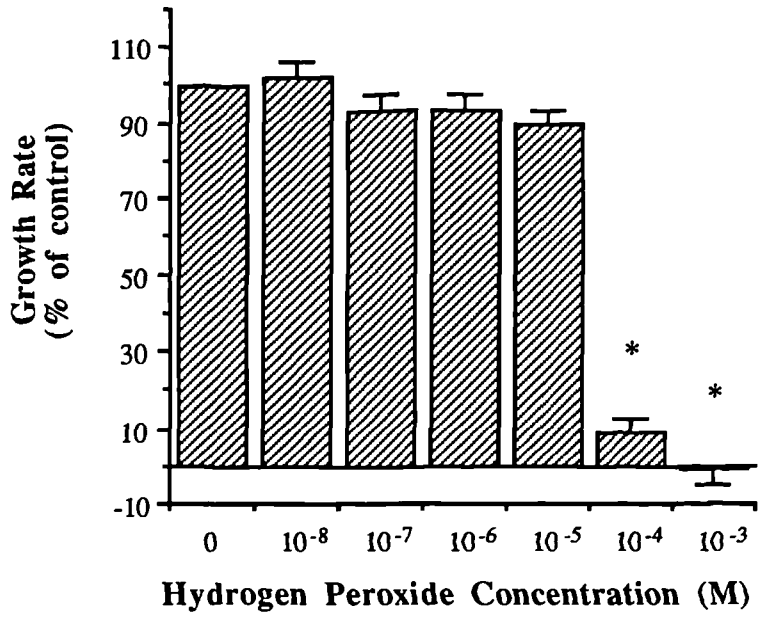


Fig. 6.5 Equine tendon fibroblast growth rates in the presence of hydrogen peroxide. Data are presented as mean ± S.E.M. n = 3 (each in quadruplicate). Statistical significance was evaluated using ANOVA. L.D.S. = 11·5%, * denotes a significant difference relative to control cells.

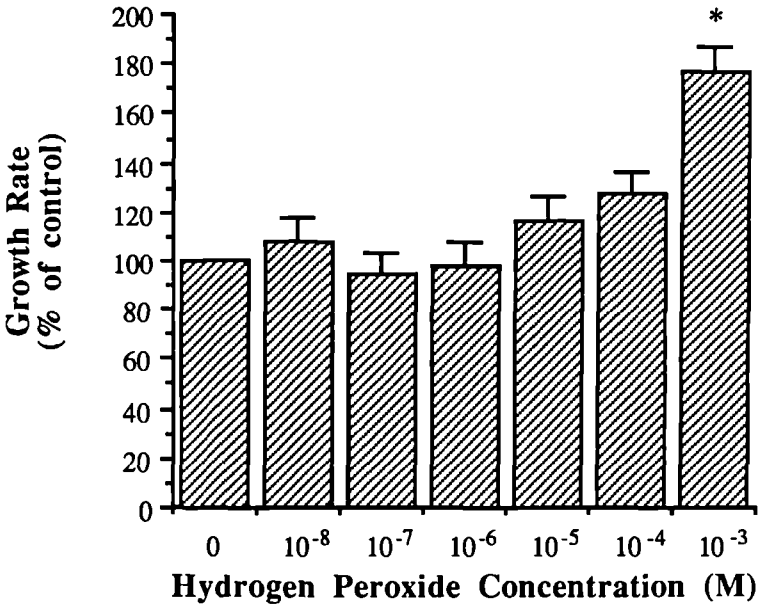


Fig. 6.6 Equine tendon fibroblast growth rates in the presence of hydrogen peroxide and free radical scavengers. Data are presented as mean ± S.E.M. n = 3 (each in quadruplicate). Statistical significance was evaluated using ANOVA. L.D.S. = 29·7%, * denotes a significant difference relative to control cells.

Collagen analysis

The banding pattern obtained when pepsin digested standard preparations of type I and III equine collagen were separated on a 7·5% gel are shown in figure 6.7. Type I collagen is non-reducible and is characterised by two polypeptide bands, the α1 (I) band and just below it the α2 (I) band. Type III collagen has one α1 (III) band which in equine collagens runs to exactly the same place on the gel as α1 (I) polypeptide. However, in the non-reduced form none of the type III collagen α chains run down the gel. This is due to a disulphide bridge forming between α1 (III) polypeptide chains in the last tripeptide before the non-helical domain of the molecule.

The antibody to type III collagen is highly specific for the α1 (III) polypeptide (fig. 6.8a) and shows no cross-reaction with either α1 (I) or α2 (I). The antibody to type I collagen binds more strongly to α2 (I) polypeptide band than to the α1 (I) band (fig. 6.8b). This antibody also picks up type I collagen contamination of the type III collagen standard and shows some cross-reaction with type III collagen peptide bands. This was not a problem in these studies as in non-reduced samples type III collagen remains at the top of the gel and therefore does not contribute to the staining for type I collagen.

Effect of FCCP and free radicals on collagen production

Hydrogen peroxide at a concentration of 10^{-6} M had no effect on secretion of either type I or III collagen into the medium (fig. 6.9 & 6.10). Incubation in the presence of 5 μM FCCP had no effect on the secretion of type I collagen (fig. 6.9). The amount of type III collagen secreted into the medium by cells in the presence of FCCP was however lower than in control cells (fig. 6.10). Autoradiographs, in experiments where radiolabelled proline was used, showed that newly synthesised collagen was present in the medium under each of the conditions studied.

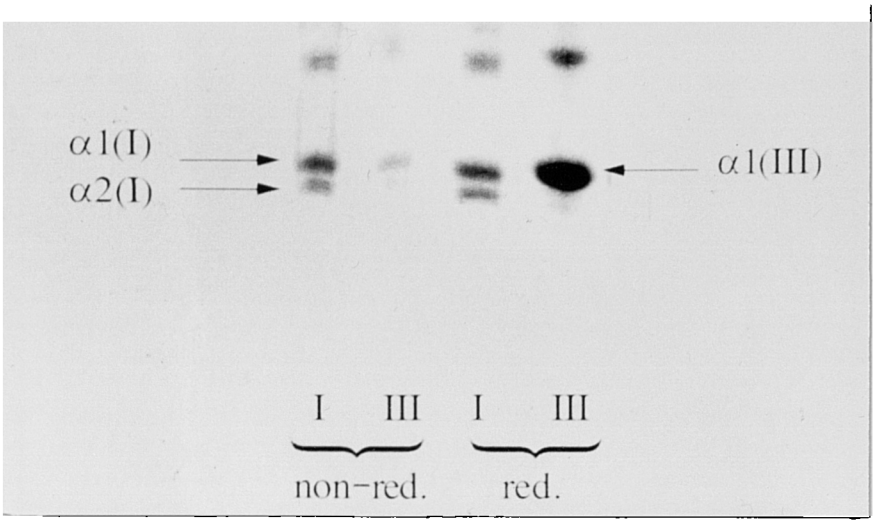
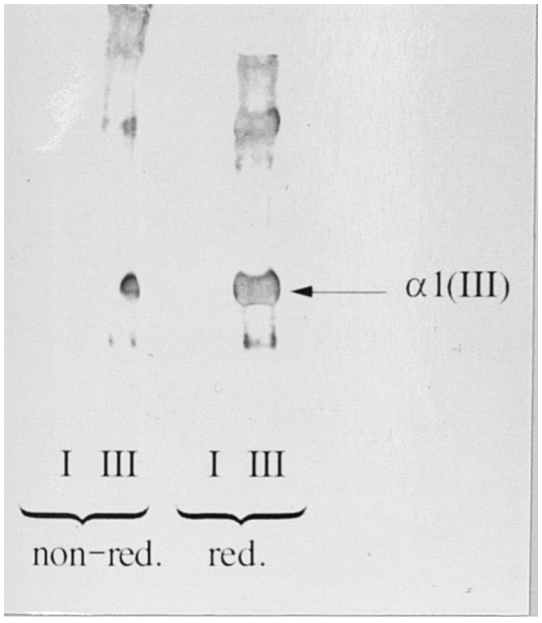
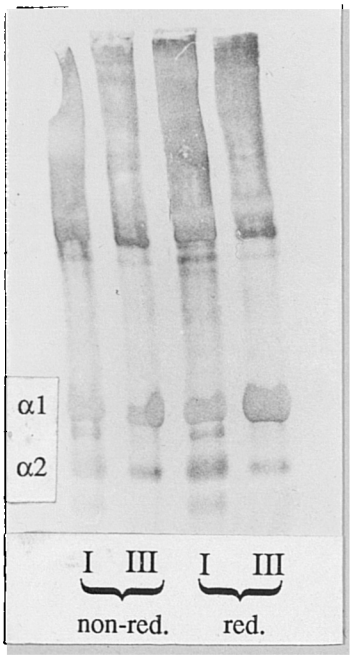


Fig. 6.7 Banding pattern obtained following separation of whole chain, pepsin digested, reduced and non-reduced, type I and III equine collagen standards by SDS-PAGE and staining with Coomassie blue.



6.8a



6.8b

Fig 6.8 Whole chain, pepsin digested, reduced and non-reduced, type I and III equine collagen standards separated by SDS-PAGE and western blotted with goat anti-type III collagen antibody (a) or goat anti-type I collagen antibody (b).

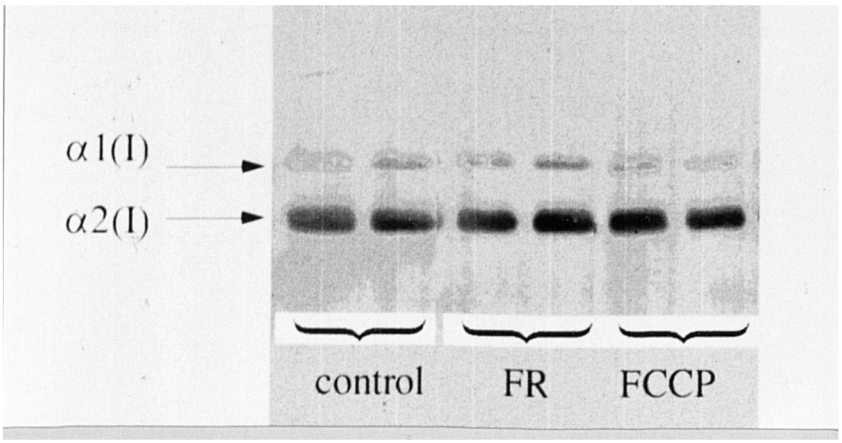


Fig. 6.9 Collagen synthesised by equine SDFT fibroblasts in the presence of 10^{-6} M H_2O_2 or 5 μM FCCP, pepsin digested, separated by SDS-PAGE and western blotted with goat anti-type I collagen antibody.

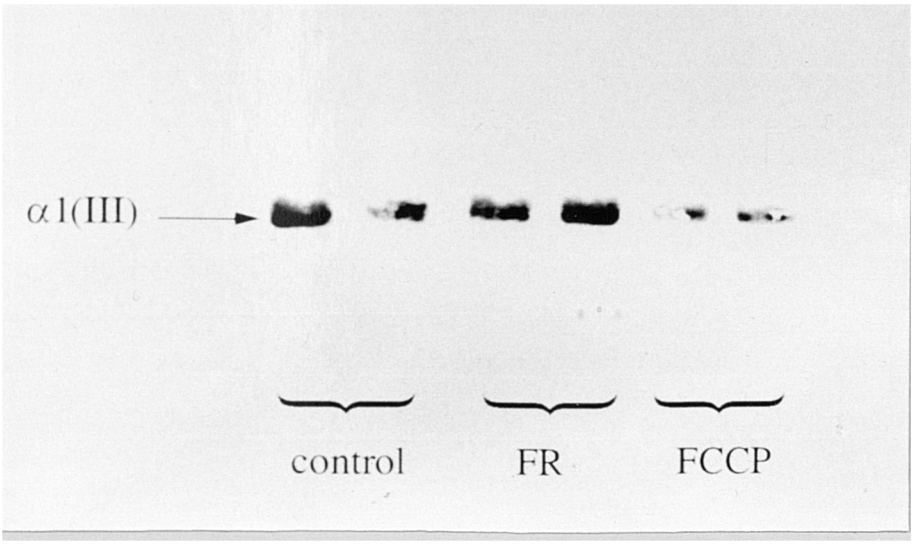


Fig. 6.10 Collagen synthesised by equine SDFT fibroblasts in the presence of 10^{-6} M H_2O_2 or 5 μM FCCP, pepsin digested, reduced, separated by SDS-PAGE and western blotted with goat anti-type III collagen antibody.

6.4 Discussion

Effect of free radicals

The generation of free radicals in the culture medium did not stimulate cell proliferation in this study. This contradicts the observations of Murrell *et al.* (1991) who found that hydrogen peroxide added to the medium (10^{-6} M) resulted in a stimulation of cell proliferation and growth in skin fibroblast cell lines. They suggested that free radical generation provides a means of rapid inter-cellular communication which might be responsible for the increase in cell density observed in some fibrotic conditions. In this study, equine SDF tendon fibroblasts showed a sharp decline in the cell survival rate between 10^{-5} and 10^{-4} M H_2O_2. If experiments were repeated with a range of concentrations between these two a linear response might be obtained or there may be an intermediate concentration at which cell proliferation is stimulated. A high concentration of free radicals ($\geq 10^{-4}$ M glyceraldehyde or $\geq 10^{-3}$ M H_2O_2) was toxic to tendon fibroblasts and resulted in cell death. Whether conditions ever occur *in vivo* where free radicals would be produced at these high levels is not known. The stimulation of cell proliferation relative to control cells (free radical scavengers but no H_2O_2) observed in this study on addition of free radical scavengers is difficult to explain. The reactions catalysed by catalase and SOD generate molecular oxygen, but the amount is small and would result in a negligible increase in oxygen tension and therefore seems unlikely to have any effect. Increase in oxygen tension following addition of 10^{-3} M H_2O_2 and free radical scavengers could be checked using an oxygen electrode in the absence of cells. The addition of hydrogen peroxide at a concentration not sufficient to cause cell growth inhibition did not affect the amount or type of collagen secreted into the medium.

Effect of hypoxia

Tendon cell proliferation rate *in vitro* did not appear to be inhibited by prevention of ATP production by aerobic pathways although cellular ATP levels are reduced (see chapter 5). FCCP at a concentration of 5 μM completely inhibits ATP production by aerobic means (Heytler & Prichard, 1962) and is therefore equivalent, in terms of energy metabolism, to complete removal of oxygen. Cells *in vitro* however, have increased capacity for anaerobic metabolism and therefore hypoxia *in vivo* may have a much more detrimental effect. FCCP at a concentration of 10 μM in these experiments is likely to be having a non-specific inhibitory effect on the cells. Neither was cell proliferation stimulated by compromising energy metabolism so this does not directly account for the apparent increase in cell density observed in degenerated

tendons. In growing tumours where cell proliferation is rapid and hence tissue oxygen tension low there is a rapid induction of lactate dehydrogenase (LDH), an anaerobic pathway enzyme, presumably to enable production of ATP in the absence of oxygen (Board *et al.*, 1990). A similar increase in LDH activity is seen in equine tendon fibroblasts under culture conditions (see chapter 5). Induction of LDH in both cases is probably due to growth factors rather than in response to low oxygen tension directly (Board *et al.*, 1990). Neither in tumour cells or in cultured cells under conditions which increase LDH activity is there any in change in mitochondrial enzyme levels and therefore aerobic capacity.

Type III collagen secretion into the medium appeared to be reduced in the presence of FCCP although type I collagen secretion was unaltered. This contradicts both the proposed increase in type III collagen attributed to low oxygen tension in degenerated tendons and the observations in wound healing where oxygen tension is low but type III collagen secretion is increased. The reason for this discrepancy is unclear. It may be that because addition of FCCP prevents cells from using oxygen for energy production but does not alter oxygen tension, unlike the true hypoxic situation *in vivo*, there is an increase in the amount of oxygen available for collagen production where molecular oxygen is a cofactor. This needs to be further investigated by culturing cells in the absence of oxygen, either in a nitrogen environment or by removing oxygen with a hydrogen and palladium catalyst, and comparing to results with FCCP.

Insufficient vascular supply to a tissue and the resultant tissue hypoxia often leads to neovascularisation, as for example in rapidly growing tumours and during wound healing (Shweiki *et al.*, 1992). The reddish staining visible in the central core of degenerated tendons may therefore be due to an angiogenic response to low oxygen tension in this region of the tendon. Vascular endothelial growth factor (VEGF) has recently been recognised as a factor which may mediate this response (Shweiki *et al.*, 1992; Plate *et al.*, 1992). In tumour specimens undergoing neovascularisation VEGF was found to be specifically induced in a subset of cells distinguished by their close proximity to hypoxic regions. A clustering of capillaries was seen alongside these VEGF producing cells. *In vitro* studies with a glioma cell line and fibroblast cell line have shown that VEGF messenger ribonucleic acid (mRNA) levels are dramatically increased within a few hours of exposure to hypoxia and return to background when normal oxygen supply is resumed (Shweiki *et al.*, 1992). Hypoxia induced production of VEGF may explain hypercapillarisation in the central core of degenerated tendons and may also provide a useful marker for identifying hypoxic regions in tendons.

In conclusion, hypoxia represents a possible cause of tendon degeneration. The results of this study show that a shift towards anaerobic metabolism alters the proportion of different collagen types produced by tendon fibroblasts. Further work is

necessary to determine the effect of low oxygen tension on tendon fibroblast synthetic activity and compare with the effect of respiratory un-couplers.

Chapter Seven

THE RÔLE OF HYPERTHERMIA IN TENDON DEGENERATION - AN *IN VITRO* STUDY

7.1 Introduction

In addition to flexion of the digit, the equine superficial digital flexor tendon (SDFT) acts as an elastic energy store during exercise. A large proportion of the energy stored elastically in straining this tendon is released upon recoil. The tendon, however, is not completely elastic, and some of the energy (about 10%, Olmos *et al.*, 1989) is dissipated as heat (Harkness, 1979) during the loading and unloading cycle. When tendon is cyclically loaded *in vitro* this energy transformation is represented by the area enclosed within the hysteresis loop (fig. 7.1). The release of heat results in the tendon warming up; heat is removed by conduction to surrounding tissue and via the blood supply.

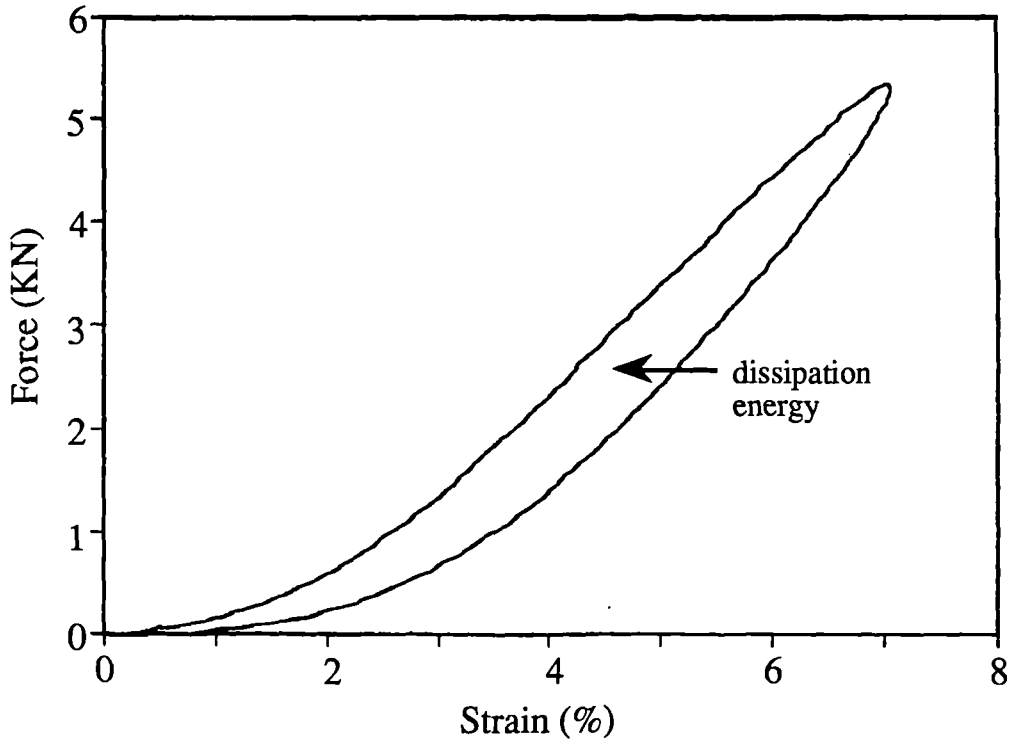


Fig. 7.1 Loading cycle of equine SDFT showing the loop of hysteresis. The area of the loop represents the energy lost in one loading and unloading cycle.

High strains, as experienced by the equine SDFT, increase the amount of heat generated whilst a poor blood supply decreases the efficiency of heat removal. It was suggested therefore that local intratendinous hyperthermia may play a rôle in equine

SDFT degeneration. It has been shown recently that a substantial temperature rise occurs in the central core of equine SDFT *in vivo* during high speed locomotion (Wilson & Goodship, 1992). Temperatures were recorded by implanting two small thermoresistors, one into the central core of the equine SDFT and the other in a subcutaneous site overlying the tendon. Recordings were made whilst the horse was exercised on a high speed equine treadmill (fig. 7.2). Temperatures as high as 45°C were recorded in the central core of this tendon during the gallop. Furthermore, mathematical models based on mechanical properties, cross sectional area and blood flow, suggest that similar rises occur in human Achilles tendon during vigorous exercise (Wilson *et al.*, 1992). Significantly, the central core of the equine SDFT, which is the site of most marked temperature rises (fig. 7.2), is also the site of degeneration and subsequent injury of both this and human Achilles tendons (Webbon, 1977, Arner *et al.*, 1959). This suggests that exercise-induced hyperthermia may play a rôle in the aetiology of degenerative change in tendon.

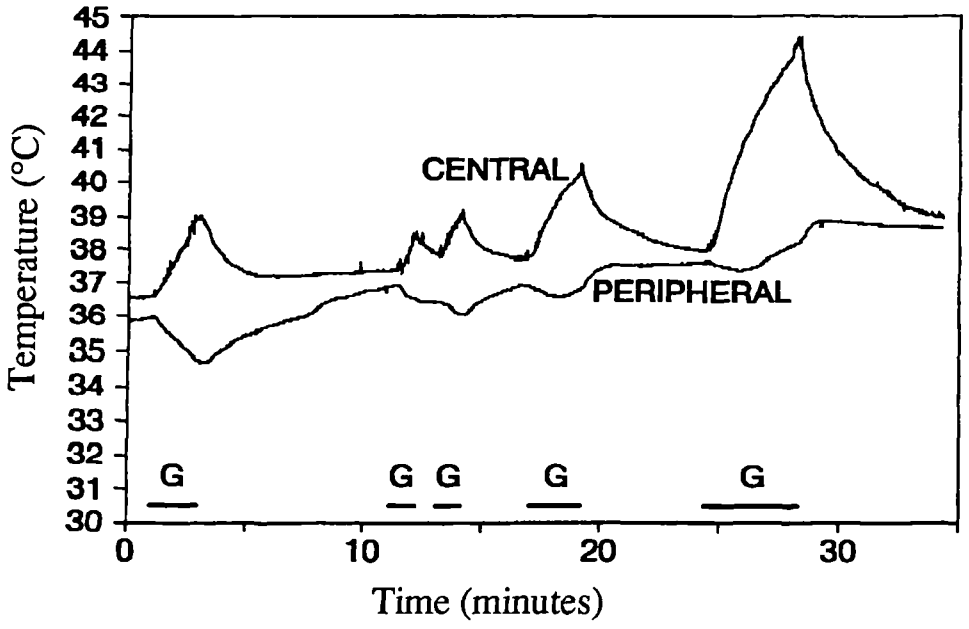


Fig. 7.2 Typical plot from a recording session with thermoresistors implanted into the equine SDFT. — represents a period of galloping. Ambient temperature 2°C. (Wilson, 1992)

A temperature of 45°C would not be expected to have a direct detrimental effect on the collagen component of the extracellular matrix. This is because, although the denaturation temperature of molecular mammalian type I and III collagen is about 39°C, when packed into the fibre, the added stability bestowed upon the collagen molecule by interactions with other collagen molecules raises the denaturation temperature to about

66°C (Kühn, 1987). This temperature is much higher than those that might be expected to occur in equine SDFT during high speed locomotion.

An alternative mechanism whereby hyperthermia could lead to tissue degeneration is through damage to the fibroblasts within the tendon. Tendon fibroblasts are responsible for maintenance of the extracellular matrix through the synthesis of collagen and other matrix components. Damage to these cells may, therefore, result in a reduction of tendon material properties. Mammalian cells from other tissues are known to tolerate increases in temperature rather poorly (fig. 7.3) (Dewey *et al.*, 1977). Severe alterations in the morphology of V79 fibroblasts are evident after exposure to a temperature of 45°C for 1 hr (Arancia *et al.*, 1989). These results suggest that the degenerative lesion observed within tendon may be the result of cellular damage secondary to localised heating during high intensity exercise.

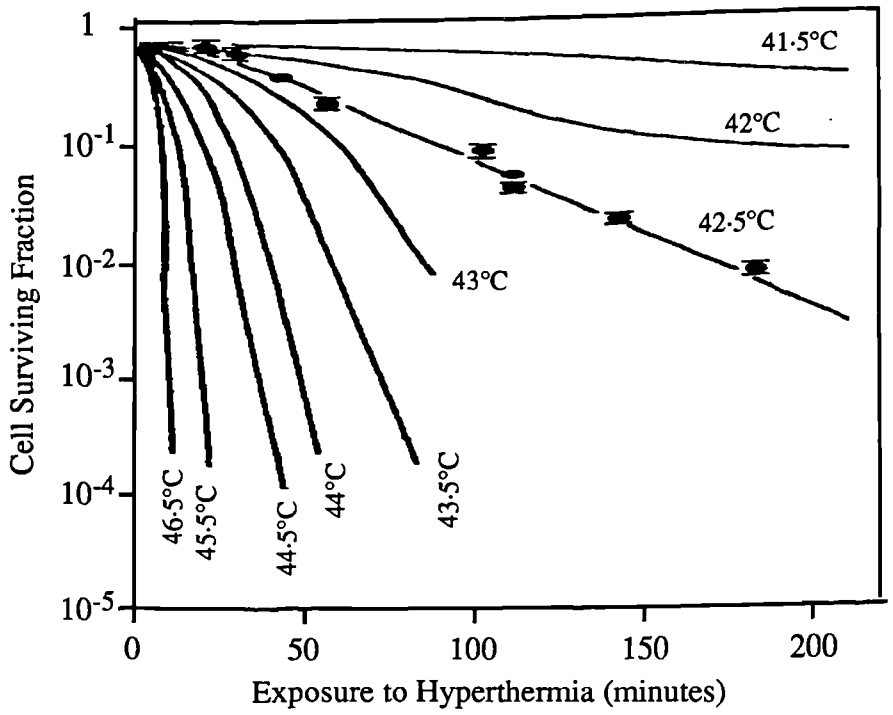


Fig. 7.3 Survival curves for mammalian cells in culture (Chinese hamster CHO line) heated at different temperatures for varying lengths of time. (Redrawn from Dewey *et al.*, 1977)

Hypothesis

Temperature rises that occur within equine SDFT during high intensity exercise will cause cellular damage which may lead to degenerative changes in the extracellular matrix.

Objectives

1. To measure equine tendon fibroblast survival rates following exposure to hyperthermia for vary lengths of time at different temperatures and compare to other fibroblast cell lines.

2. To assess the effect of a previous "heat shock" or un-coupler of oxidative phosphorylation on equine tendon fibroblast survival levels following exposure to hyperthermia.

3. To determine the effect of a "heat shock" on equine tendon fibroblast growth rates and collagen secretion.

Experimental design

Lethal cell damage following exposure to hyperthermia can be assessed by measuring the cell fraction that is able to re-establish under culture conditions. Other methods, such as trypan blue exclusion, are not as reliable as cells may appear to be viable immediately following exposure but subsequently are unable to survive. Cellular adenosine triphosphate (ATP) level is a sensitive marker of cellular state (Baur *et al.*, 1975) and this was measured as an indicator of cellular stress and sub-lethal damage which may occur during the heating process. Also, a failure of energy metabolism and fall in cellular ATP levels may represent a pathway for lethal cell damage during periods of hyperthermia. A temperature of 45°C was chosen for the above experiments as this was the highest temperature recorded *in vivo* and the time of incubation was extended until the cell survival fraction fell to below 10%. The period of time for which a horse could gallop at a speed great enough to produce temperature rises of any magnitude would not exceed 10 min. Further studies, therefore, were conducted where exposure to hyperthermia was limited to 10 min. but a range of temperatures from 37°C to 54°C were used. Hypoxia may increase the amount of cell damage caused by hyperthermia and this was simulated in these experiments by inclusion of an un-coupler of oxidative

phosphorylation (FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone) in the medium. The situation *in vivo* would relate to a series of "heat shocks" which may effect sensitivity to subsequent exposures, or, whilst not lethal to the cell may alter cell proliferation rates and collagen production. These possibilities were investigated in equine tendon fibroblasts following a 10 min. heat shock at 45°C.

7.2 Methods

Cell culture

Explant-derived tendon fibroblasts from the central core of normal equine SDFT were grown in culture as described in chapter 5. In experiments where skin fibroblasts were used as a comparison to tendon fibroblasts, these were grown in exactly the same way from explants of skin taken from the region overlying the SDFT (see appendix 2). Further comparison was made with a commercially available rat kidney fibroblast cell line (NRK 49F). All cells were maintained in monolayer culture in DMEM (Dulbecco's modified Eagle's medium) buffered with 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) plus 10 % foetal calf serum (FCS), antibiotics (streptomycin, 100 µg/ml; benzylpenicillin, 200 U/ml; neomycin, 50 U/ml) and fungizone (Amphotericin, 5 µg/ml), in a humidified atmosphere containing 5% CO_2. Experiments were carried out on cells between passages 3 and 11.

Exposure to hyperthermia

The effect of hyperthermia on tendon cell survival rate was assessed by heating cell suspensions in a waterbath for varying lengths of time. Suspensions of tendon fibroblasts were obtained by trypsin digestion (using 0.05% trypsin in sterile phosphate buffered saline (PBS) containing 0·02% EDTA (diaminoethanetetra-acetic acid) and 0·01% phenol red). After washing, suspensions of cells (0·5 x 10^6 cells/ml) in medium supplemented as above, were immersed in a waterbath and maintained at 45°C in 7 ml tubes (Sterilin) for periods of 0 - 180 min. After heating, cells were transfered back into culture flasks, fresh medium added and flasks returned to the 37°C incubator for 36 hours during which time viable cells were able to re-attach to the surface of the flask. The experiment was repeated with 4 different tendon fibroblast cell lines (i.e. cells grown from 4 different horses). As a comparison, experiments were repeated in the

same way using equine skin fibroblasts (n = 3) and a commercially available rat kidney fibroblast cell line (NRK 49F, n = 4).

Quantification of cell survival

Viable cells adherent to the bottom of the flask were quantified by a deoxyribonucleic acid (DNA) assay, following papain digestion (Kim *et al.*, 1988). Prior to digestion, the medium was decanted and the flasks rinsed thoroughly with sterile PBS to remove damaged unattached cells. Results were expressed as % cell survival relative to those cells kept at 37°C.

ATP levels during periods of incubation at 45°C

Cellular ATP levels in tendon fibroblasts were measured following exposure to hyperthermia. Heating was carried out at 45°C for time periods of 0 - 180 min. as above. Confluent tendon fibroblasts were trypsin released, washed in buffer and resuspended in supplemented medium. Incubations were set up with 1 ml of cell suspension and either kept at 37°C for times of 0 - 180 min. or incubated at 45°C for times of 10 - 180 min. (in duplicate). Experiments were repeated 3 times with a different cell lines. After the appropriate time the reaction was stopped by the addition of 70% perchloric acid (15 µl) and frozen immediately on dry ice. At the end of the experiment samples were thawed and neutralised with 5 M KOH, 100 mM HEPES and 2 mM EDTA. Sediment was removed by centrifugation before assay of ATP.

ATP assays

ATP assays were carried out essentially as done by Stanley & Williams (1969) using firefly luciferase and flashes of light detected with a photomultiplier in a liquid scintillation counter. The reaction buffer consisted of sodium arsenate (38 mM), sodium phosphate (7·7 mM) and $MgSO_4$ (16·8 mM) and was prepared immediately before use. Reaction buffer (2 ml) was placed in plastic vials, 50 µl of cell suspension was added to each vial; blanks were prepared by adding 50 µl of cell suspension alone. The reaction was started by the addition of 10 µl of luciferase and counting was performed over 30 s. Levels of ATP in the experimental samples were calculated from a standard curve (10 - 500 pmol ATP).

Effect of hypoxia or previous "heat shock" on cell survival fraction

Experiments were carried out essentially as above. A fixed time of one hour was used at a temperature of 45°C. Hypoxia was simulated by the addition of 5 μM FCCP (sufficient to completely un-couple oxidative phoshorylation, Heytler & Prichard, 1962) to the medium immediately before heating the cells. Controls were conducted by heating cells from the same suspension in the same way in the absence of FCCP. Following heating both control and experimental cells were resuspended in fresh medium before returning to the incubator for re-establishment. Experiments were repeated 3 times using 3 different cell lines each in triplicate.

A 10 min. heat shock was administered while cells were adherent to the bottom of a culture flask. Following the heat shock cells were supplied with fresh medium and returned to the incubator. Exposure to hyperthermia for 1 hr at 45°C was carried out 48 hr later as described above. Control cells which had received no previous heat shock were suspended in medium to an identical concentration and heated alongside. Cell quantification was carried out as above. Cell survival rates were expressed relative to the control cells heated for 1 hr at 45°C. Experiments were repeated using 3 different cell lines each in duplicate.

Effect of temperature on cell survival fraction

The time for which a horse might be galloping at a speed great enough to produce a temperature of 45°C would not exceed 10 min. For this reason experiments on cell survival following exposure to hyperthermia were repeated using a fixed time of exposure (10 min.) and a range of temperatures (37 - 54°C). Experiments were carried out on tendon fibroblasts (n = 3) and skin fibroblasts (n = 3) by the method described above and cell survival quantified in the same way.

Growth rates

Cell proliferation rates were measured, using the experimental method described in chapter 6, following a 10 min. "heat shock" at 45°C. "Heat shock" was carried out 24 hr prior to the addition of radiolabelled thymidine. Experiments were repeated 4 times in quadruplicate.

Collagen production

Collagen secretion into the medium was measured following a "heat shock". The conditions used for collagen production and the method of collagen analysis were as described in the previous chapter. Heat shock was given to the cells at 45°C for 10 min. 12 hr after the start of incubation or, in experiments where collagen synthesis was followed by the incorporation of proline (5 μCi of [U-^{14}C]-proline, specific activity 260 Ci/mol), immediately before the addition of proline. Flasks were returned to the 37°C incubator for 24 hr. Experiments were repeated 5 times using 3 different cell lines.

Statistical analysis

Statistical significance was evaluated using ANOVA. Data are given as mean ± S.E.M. Level of significance was taken as $p \leq 0{\cdot}05$.

7.3 Results

Effect of hyperthermia on cell survival fraction

Cell survival fractions following exposure to hyperthermia (45°C) for times of 0 - 180 min. are shown in figure 7.4. Explant-derived fibroblasts from the equine SDFT display a remarkable resistance to elevated temperature and even after 30 minutes of heating the fraction of cells able to re-establish and grow was not significantly reduced. After 60 minutes of heating approximately 25% of tendon cells were irreversibly damaged and unable to survive, representing a significant reduction. Skin fibroblasts and NRK49F cells appeared to be more sensitive to hyperthermia and at time points 10 min. and 20 min. respectively cell viability was significantly reduced. After 60 minutes of heating less than 25% of cells remained able to re-establish and grow in culture.

Cellular ATP levels were not significantly reduced during the course of the experiment (fig. 7.5). This suggests firstly, that cell death was not caused by a failure of energy metabolism, and secondly, that cell death was not immediate but must occur at a later time. This is because cell death would result in leakage of cellular contents into the medium and a rapid hydrolysis of ATP. The mean value for cellular ATP levels measured in each of the three experiments were $4{\cdot}82 \pm 0{\cdot}07$, $6{\cdot}24 \pm 0{\cdot}14$ and $4{\cdot}81 \pm 0{\cdot}07$ pmoles ATP/10^3 cells.

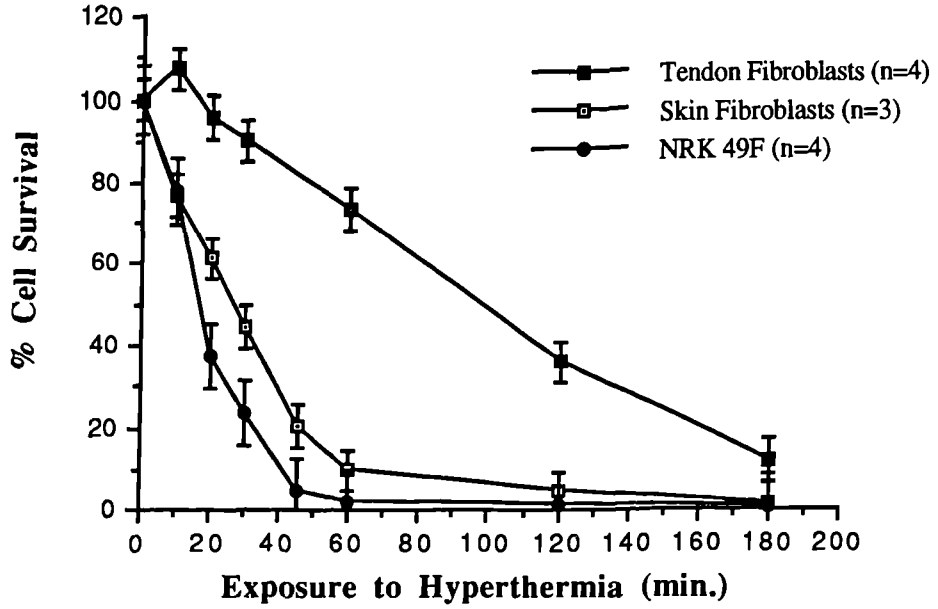


Fig. 7.4 Cell survival following exposure to hyperthermia at 45°C.

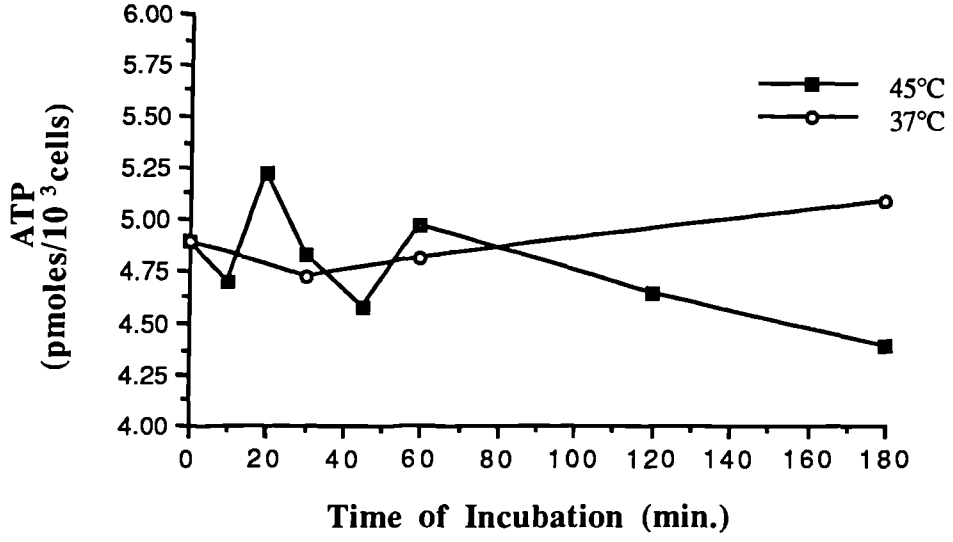


Fig. 7.5 Cellular ATP levels following exposure to hyperthermia (45°C).

Using a fixed 10 minute time of incubation a sharp decline in the cell survival fraction was seen between 47 and 49°C in equine tendon fibroblasts (fig. 7.6). Equine skin fibroblasts showed a sharp decline in survival within the same temperature range (fig. 7.7).

Effect of hypoxia or previous "heat shock" on cell survival fraction

Heating in the presence of FCCP, an un-coupler of oxidative phosphorylation, did not alter the cell survival rate, whilst a "heat shock" prior to exposure to hyperthermia significantly increased cell survival rates by about 10% ($n = 3$, $p < 0{\cdot}001$) (table 7.1).

Table 7.1 Equine SDFT fibroblast survival fraction following exposure to 45°C for one hour under different conditions. Hypoxia was simulated by the presence of 5 μM FCCP (n = 3). Heat shock was given at 45°C for 10 min. 48 hours before the start of the experiment (n = 3).

Treatment	Cell Survival
Heat shock	110 ± 1 *
FCCP	100 ± 1

Cell growth rate

"Heat shock" stimulated proliferation of tendon fibroblasts relative to the controls in all four experiments (each in triplicate) with cells grown from three different horses (127% ± 8, $p = 0{\cdot}02$, $n = 3$).

Collagen production

Heat shock increased the secretion of type I collagen (fig. 7.8) and type III collagen (fig. 7.9) into the medium in all 5 experiments.

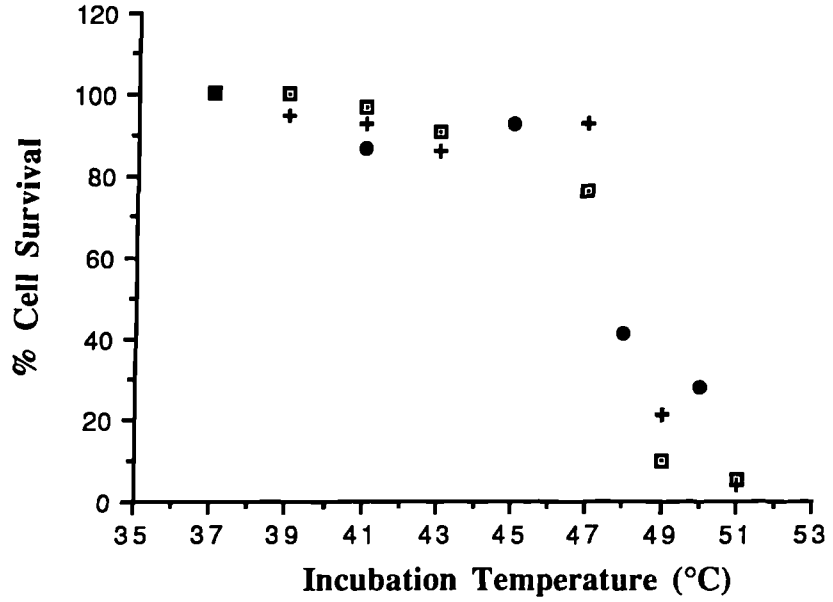


Fig. 7.6 Equine tendon fibroblast survival following exposure to hyperthermia (10 min.). Different symbols represent tendon fibroblasts grown from different horses.

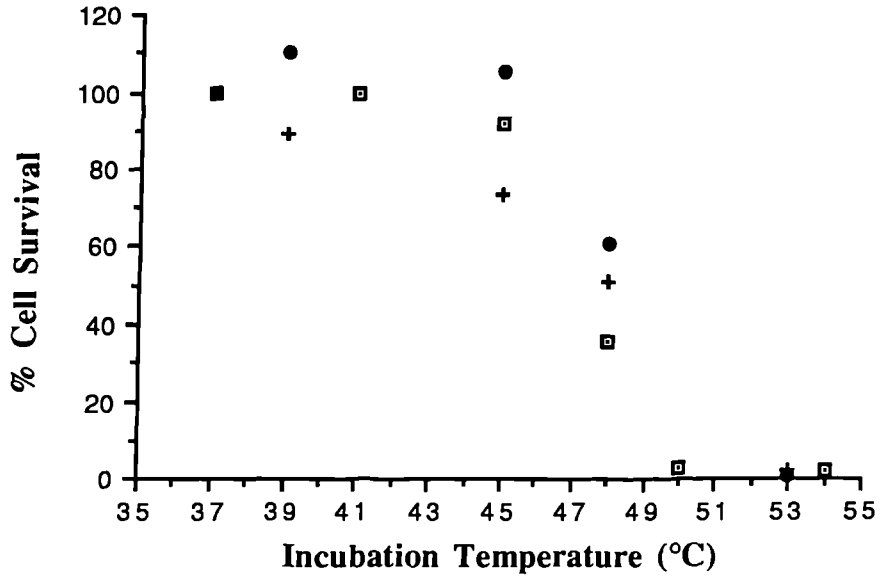


Fig. 7.7 Equine skin fibroblast survival following exposure to hyperthermia (10 min.). Different symbols represent skin fibroblasts grown from different horses.

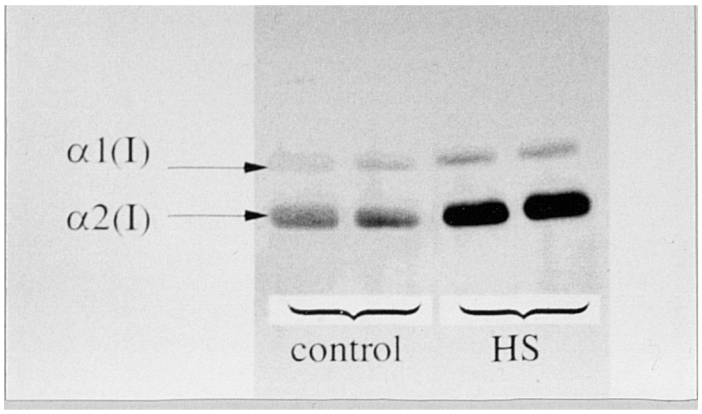


Fig. 7.8 Collagen synthesised by equine SDFT fibroblasts following a 10 min. heat shock at 45°C, pepsin digested, separated by SDS-PAGE and western blotted with goat anti-type I collagen antibody.

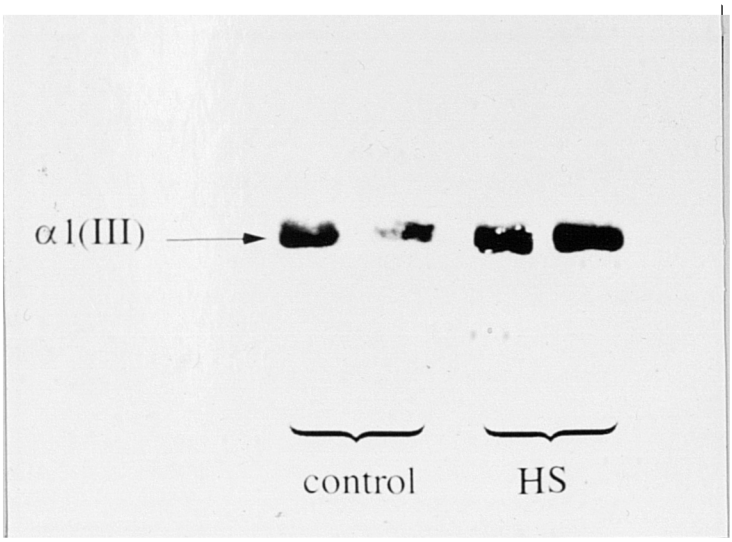


Fig. 7.9 Collagen synthesised by equine SDFT fibroblasts following a 10 min. heat shock at 45°C, pepsin digested, reduced, separated by SDS-PAGE and western blotted with goat anti-type III collagen antibody.

7.4 Discussion

Cell survival following exposure to hyperthermia

Fibroblasts cultured from the central core of equine SDFT have an increased resistance to hyperthermia compared to a commercially available rat kidney fibroblast cell line (NRK 49F). The possibility that this was an adaptation due to the way in which the cells were grown in culture was eliminated by comparison with equine skin fibroblasts, which had been collected and grown in an identical manner. Equine skin fibroblasts displayed a similar sensitivity to hyperthermia as that of the rat kidney fibroblasts. Therefore, thermal resistance appears to be an inherent characteristic of the tendon fibroblasts. The ability to withstand a temperature of 45°C would be advantageous for cells in the equine SDFT and demonstrates an adaptation for their physiological environment. Furthermore, degenerative changes to the extracellular matrix (see chapter 3) were not associated with a decline in cellularity and cell activity. Whilst these results suggest that temperatures reached within the central core of the SDFT during the gallop are neither high enough or sustained long enough to cause irreversible cell damage, differences in cell environment during the heating process must be taken into consideration. Matrix to cell and cell to cell interactions which would exist *in vivo* are lost when cells are heated as a suspension *in vitro* (Freshney, 1987) Also, during these experiments cells were heated in a culture medium containing all the essential nutrients and buffered against changes in pH. In an *in vivo* situation cellular environment may be altered during the period of hyperthermia in any of the following ways; nutrient deprivation, drop in oxygen tension and pH, and increase in lactic acid concentration. There are reports that cells respond differently to thermal stress under different conditions. Hypoxia increases thermal sensitivity (Dewey *et al.*, 1977) although this may only be true of cells subjected to chronic hypoxia (Hall, 1988). Inclusion of FCCP in the medium during heating in these studies simulates a period of acute hypoxia and this had no effect on cell survival levels. It may be that if the cells were subjected to chronic hypoxia thermal sensitivity would be increased. Glucose deficiency and a low pH may also increase hyperthermic damage (Hall, 1988). Further *in vitro* experiments are necessary to determine the effect of heating whilst depriving cells of nutrients and/or lowering the pH of the medium.

As a temperature of 45°C for a duration of 10 min. did not appear to result in cell death a range of temperatures were used for the same duration on both tendon and skin fibroblasts. In these studies a temperature of 48°C appeared to be the point, for both cell lines, at which irreversible cell damage occurred and a substantial drop in the cell survival fraction was seen. It is possible that in extreme cases a temperature of 48°C may be reached in the equine SDFT during racing and this would be expected to result

in cell death. By comparison, skin fibroblasts, even on a hot day in direct sunlight, are unlikely to experience temperatures above 40°C. This is because the dermis is a very good thermal insulator and also skin has a rich blood supply which will dissipate any heat.

Cellular thermotolerance is not a new concept, although it is usually associated with cells that are genetically endowed for existence at high temperatures, such as thermophilic algae and bacteria, rather than mammalian cells. Some of these species may be found in hot springs at 60 - 70°C and grow only with difficulty at lower temperatures (Harris, 1967). In some cases bacteria are able to grow at a temperature higher than that which causes cell damage (Mackey *et al.*, 1993) and therefore a constant repair process must be taking place. Differences in thermotolerance has also been found between other cell lines (Auersperg, 1966) for example pig kidney cells are more than 10 times more resistant to a temperature of 46°C than Chinese hamster ovary cells (Dewey, 1977). Thermotolerance can also be induced by exposure of cells to a sub-lethal temperature (Harris, 1967; Harisiadis *et al.*, 1977). The mechanism behind this apparent acquired thermal resistance following exposure to sub-lethal temperatures remained un-resolved for some time but was not attributed to selection of a thermoresistant sub-population of cells (Harisiadis *et al.*, 1977). These initial observations may now be explained by the presence of the recently discovered group of proteins known as "heat shock proteins". This small set of evolutionary conserved proteins are expressed following a shift to a higher temperature, or indeed to any other cell stress (Craig & Gross, 1991) and protect the cell from further insults for several days (Hall, 1988). The increased resistance of tendon fibroblasts to hyperthermia observed in this chapter following a previous heat shock may be due to expression of these proteins. This could be determined by subjecting cells to a heat shock, separating intracellular proteins by electrophoresis and using immunodetection techniques to look for heat shock proteins. Heat shock proteins are highly conserved between species and antibodies are commercially available.

There are several possible targets for heat induced damage and different mechanisms may be responsible at different temperatures (Hall, 1988). This would explain the similar response by tendon and skin fibroblasts to a temperature of 48°C, while differing in sensitivity to a temperature of 45°C. Homeostasis of cellular ion concentrations is critical for cell survival. Pumping of sodium, potassium and calcium ions occurs against a concentration gradient and is therefore an active process requiring ATP (Vander *et al.*, 1985). An increase in temperature increases the diffusion of ions along their concentration gradients and hence there is an increased demand for ATP. Failure of metabolic pathways to maintain ATP levels might therefore be a possible cause of cell damage. In these experiments cellular ATP levels were not significantly reduced even after 180 min. at 45°C and so this does not seem like a possible cause of

damage. Further evidence that cell death does occur due to a failure to maintain ATP levels is provided by the lack of effect of FCCP on cell survival levels. The fact that ATP levels do not decline to the same extent as cell survival levels suggests that cell death did not occur immediately but at some later point. This is because cell death would result in leakage of cellular contents into the medium and a rapid hydrolysis of ATP. A similar finding was reported by Auersperg (1966), where cell damage reached a maximum 2 - 3 days after heating. Other possible targets for thermal damage include the plasma membrane, proteins and DNA (Hall, 1988); damage to these components is likely to be lethal to the cell.

Temperatures much higher than those of interest in this study are necessary before denaturation of collagen within the extracellular matrix takes place. Disruption of other non-collagenous molecules such as proteoglycans though not investigated in this study can not be disregarded. Such effects may alter the strength of the matrix through an effect on matrix organisation.

Effect of a "heat shock" on cellular growth rate and collagen production

The response of tendon fibroblasts to a single heat shock at 45°C for 10 min was to increase cellular activity. Growth rates were significantly increased by about 25% and secretion of both types I and III collagen into the medium enhanced. The effect of heat shock on collagen production may be exerted at one or more of several points in the sequence of events leading to appearance of collagen extracellularly. Heat shock may increase transcription, which could be detected by an increase in messenger ribonucleic acid (mRNA) levels, increase translation of mRNA, decrease intracellular degradation of newly synthesised collagen or increase secretion of synthesised collagen into the medium. Further experiments are necessary to determine at which point control is exerted.

In conclusion, sub-lethal periods of exposure to elevated temperature may be responsible for both the high cellularity and increased collagen turnover observed in degenerated tendons (chapter 3). Furthermore, hyperthermia will increase cellular demand for oxygen by increasing the rate of cell metabolism thereby increasing the potential for ischaemic damage.

Chapter Eight

GENERAL DISCUSSION

8.1 Introduction

The data presented in this thesis demonstrate a number of novel and interesting biochemical findings which may provide parameters to influence the future development of training programmes and treatment regimes for tendon injuries in human and equine subjects.

It has previously been reported that a degenerative change, characterised by a reddish staining in the central core of the tendon, occurs prior to tendon rupture (Webbon, 1977) but the changes to the extracellular matrix at a molecular level and the cellular mechanisms involved in this change had not been studied. Results of the studies in this thesis show that the macroscopic appearance of these degenerated tendons is associated with an increase in collagen turnover, increased type III collagen content, increased total glycosaminoglycans and high tendon cellularity in the central core of the tendon.

In vitro simulation of the localised temperature rise that occurs in the superficial digital flexor tendon (SDFT) during high speed locomotion showed a stimulation of cell proliferation and production of type I and III collagen in cultured tendon fibroblasts. Furthermore, equine SDFT fibroblasts displayed an increased hyperthermic resistance relative to other fibroblast cell lines. The effect of hypoxia through generation of free radicals or inhibition of aerobic metabolism showed an effect on cultured tendon fibroblasts when simulated *in vitro*. Free radicals at high concentrations were toxic to cells and resulted in a dramatic loss of viability but at a concentration not sufficient to cause cell death had no effect on collagen production. Inhibition of energy metabolism by aerobic pathways did not cause cell proliferation but did reduce the production of type III collagen without altering type I collagen production.

8.2 Aetiology of Tendon Degenerative Change

Possible rôle of free radicals in tendon degeneration

A possible cause of damage to tendon considered in this thesis was free radical mediated damage following ischaemia and reperfusion. The concentrations of free radicals used in these studies, however, did not stimulate cell proliferation and so would not directly account for the high cellularity observed in degenerated tendons. Further evidence for reperfusion injury could be obtained by looking for markers of free radical damage such as iron deposits in sections of tendon from horses destroyed

on the racecourse i.e. after severe exercise. It may be possible to protect from reperfusion injury by giving allopurinol prior to racing. Allopurinol is an inhibitor of the enzyme xanthine oxidase (Parks & Granger, 1986) and so prevents the formation of free radicals by this pathway. Alternatively, high levels of free radical scavengers such as vitamin E could be fed in an attempt to scavenge free radicals (Witt *et al.*, 1992).

Possible rôle of hyperthermia in tendon degeneration

The results of chapter 7 show that hyperthermia is a particularly interesting factor with respect to tendon degeneration. This is because temperature rises are confined to the central core of the tendon (Wilson & Goodship, 1992), the region which shows degenerative changes. Furthermore, hyperthermia simulated in a culture system result in stimulation of both cell proliferation and collagen production. "Heat shock" therefore may be the stimulus for increases in matrix turnover.

Several factors will contribute to the temperature reached in the central core of the tendon during exercise. High tendon strain will increase the amount of heat generated (Wilson, 1991). Exercising horses at a slower speed and increasing work load by training horses up a gradient may have a protective effect by reducing heat generation. Insulating the leg prevents air flow cooling the peripheral tissue therefore making it less efficient as a heat sink. When being ridden horses often wear protective boots covering the leg from below the knee to just above the fetlock protecting the tendon from an external blow. This practice may well increase the potential for hyperthermic damage.

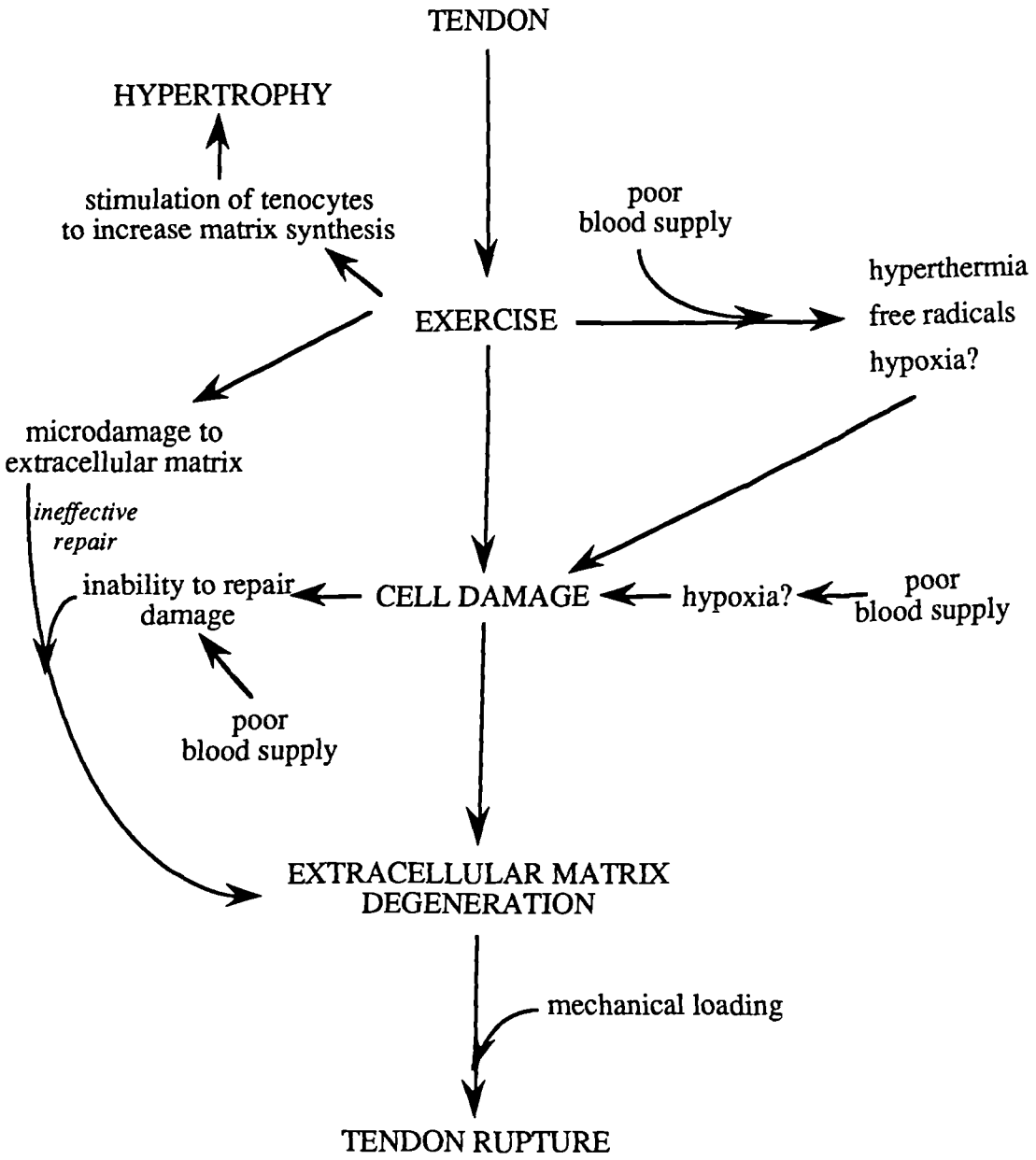


Fig. 8.1 Factors which may affect cell function leading to degenerative change.

Possible rôle of degradative enzymes in tendon degeneration

The collagen-linked fluorescence results in chapter 3 indicated an increase in collagen turnover in the central core of degenerated tendons. This implies that there was an increase in collagen degradation although this was not studied specifically in these experiments. There may, however, be an imbalance of synthesis and degradation, in favour of degradation, which would not be not picked up by the measurements made in these studies. This is because release of collagenase and cathepsins may result in a

limited degradation of collagen molecules whereby the collagen molecule is cleaved at points along its length but is not released from the matrix. This would result in a considerable weakening of the tendon but no change in collagen content. Recent *in vitro* studies have shown that hydrostatic pressure on cartilage chondrocytes results in an increased production of cathepsins (Maciewicz *et al.*, 1991). Cells in the central core of equine SDFT may also respond to increased pressure during mechanical loading and an increased number of mechanical loading cycles could result in an elevated production of cathepsins. Temperature rises in the central core of the tendon may be responsible for a higher enzyme activity in this part of the tendon. This possibility could be investigated in a group of trained versus control horses using antibodies for the detection of cathepsins.

It seems likely that degenerative changes in tendon are caused by a combination of several factors. Excessive levels of strain in the tendon by, for example, galloping overweight horses and galloping downhill will increase the amount of heat generated within the tendon. Blood flow may also be decreased further in some individuals by dissymetry of locomotive action (Dow *et al.*, 1992) and a wringing out effect on the tendon (Smart *et al.*, 1980). Decrease in blood flow may result in tissue hypoxia and this would be accentuated by an increase in metabolic rate due to temperature rises all of which may result in a change in cell function. Repetitive loading cycles may cause matrix microdamage and, due to the change in cell function, an inappropriate repair response resulting in tendon degeneration. Alternatively, some of the factors discussed above may directly trigger an increase in collagen turnover by tenocytes. The reason, however, why increased turnover *in vivo* and also the repair process following microdamage or gross damage does not result in production of a matrix composition identical to the pre-existing matrix is not clear, but may well be due to metabolic compromise.

8.3 Matrix Metabolism Studies

In chapters four and five it was shown that tenocytes have mitochondrial enzymes and carry out oxidative metabolism, despite reports to the contrary in the published literature (Landi *et al.*, 1980a; Floridi *et al.*, 1981). Furthermore, explant derived SDFT fibroblasts depended upon oxidative metabolism to maintain cellular ATP levels. The reports of tendon metabolic inertia have come from studies where collagen turnover was measured (Neuberger *et al.*, 1951; Thompson & Ballou, 1956; Gerber *et al.*, 1960). These studies were carried out on rats, a short-lived species and results cannot necessarily be extrapolated to longer lived species and to tendons that work within narrow safety margins (Stephens *et al.*, 1989). Collagen turnover in

tendons (> 110 days for rat tail tendon; Gerber *et al.*, 1960) is undoubtedly low compared with intracellular proteins and other extracellular proteins and also compared with the collagen component in other tissues such as bone (40 days), intestine (20 days) and liver (30 days) (Gerber *et al.*, 1960). It must be remembered, however, that cellularity of tendon is very low relative to the amount of extracellular matrix and thus, activity of each single cell may be much higher than first expected.

An accurate measurement of collagen synthesis in the SDFT would be of great value in furthering the understanding of tendon degeneration. The degree of glycation gives some idea of the collagen turnover rate relative to other tissues in the body but this will also depend upon the level of glucose to which the tissue is exposed. The best way to produce precise data on collagen turnover rates in the SDFT would be to label the collagen with radioactive proline. However, this is not feasible in a large animal such as the horse due to the large amount of radiolabelled proline which would be necessary to label the collagen sufficiently, and the associated cost.

Another possible way to obtain information on collagen turnover rates would be to work with primary explants of tissue in a culture system. In this way, cell-matrix interactions are maintained. Collagen synthesis could be studied within the first 24 hr following tissue removal from the horse and would give an indication of collagen synthesis rates *in vivo*. The problems of diffusion and nutrient deprivation which are encountered when working with un-disaggregated tissue in culture must not be overlooked. Any differences in collagen synthesis rates between trained and control horses and between central and peripheral zones of the same tendon could be further verified by in situ hybridisation. This technique enables the degree of transcription of type I and III collagen genes to be measured by probing for type I and type III collagen messenger RNA (mRNA) on tendon sections.

Synthesis and degradation of proteoglycans is also of interest, particularly considering the differences observed between the DDFT and SDFT in chapter two and between normal and degenerated SDFT in chapter three. Proteoglycan synthesis could also be studied over a short time period in an explant culture system to get an estimate of turnover rates *in vivo*.

8.4 Cell Culture Studies

The use of cultured cells enables longer term experiments to be carried out under controlled conditions. These systems are ideal for looking at the effects of different culture conditions and drugs on tendon cell metabolism including collagen and proteoglycan synthesis. There are, however, important differences between the *in vivo* and *in vitro* situation and care must be taken when relating results obtained *in vitro* to

the whole animal. The main difference is that tissue integrity and therefore cell-matrix interactions are lost in cell culture experiments and biomechanical stimulus is removed. The biochemical surroundings are also altered when cells are grown in culture such that an excess of nutrients are supplied in the culture medium, but oxygen tension, which depends on diffusion through the medium will be reduced at the cell surface. Another important difference to consider is that cells in culture are growing rapidly whereas those in intact mature tendon are not, except for example in the first stages of the repair process, and this may alter cellular function. Cell proliferation is stimulated in culture by the addition of foetal calf serum, which contains inherent growth factors. Inclusion of either foetal calf serum or supplementation with another source of growth hormones is essential to grow cells in a culture system. In further studies it may be possible to prevent rapid cell growth by either complete or partial removal of the foetal calf serum from the medium. Once cells had become established in a flask and adequate cell numbers obtained foetal calf serum could be removed and matrix metabolism studied in a non-dividing population of cells. By pulse labelling, the synthesis and degradation of collagen could be studied over a time course on the same cells following different stimuli.

Simulation of hypoxia in vitro

The effect of hypoxia on cell function was studied in this thesis by the addition of a respiratory un-coupler to the culture medium. This prevents the cell from using oxygen to produce adenosine triphosphate (ATP) and mimics the effect low oxygen tension would have on energy metabolism. Oxygen, however, is still present in this system unlike a true hypoxic situation *in vivo*. Molecular oxygen is required for collagen synthesis as it is a cofactor for both proline hydroxylase and lysyl hydroxylase and is still available for this purpose even after addition of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). The effects of FCCP on collagen secretion (decrease type III, type I unchanged) seen in this thesis are purely due to the effect of hypoxia on energy metabolism. Actual removal of oxygen from the system may have a different effect. This could be achieved either by completely removing oxygen using hydrogen and a palladium catalyst (Shweiki *et al.*, 1992) or by gassing the culture incubator with nitrogen and thereby creating low oxygen tension. This would have to be monitored using an oxygen electrode. Collagen synthesis could then be compared in cells cultured in low oxygen tension and cells cultured in the presence of FCCP. Any conditions applied *in vitro* must be a simulation of those which occur *in vivo*, one of the problems with this part of the study is that to date no measurements of oxygen tension have been made *in vivo*. This is because measurements are difficult due to the

nature of the oxygen electrode; calibration is difficult, the electrode is poisoned rapidly when in the tendon and movement artefacts also occur.

Investigation of thermal resistance in equine SDFT fibroblasts in vitro

In chapter seven it was shown that tenocytes are particularly resistant to hyperthermia. The reason for this increased resistance relative to other fibroblast cell lines is not clear. It has been known for a long time that different cell types respond differently to increased temperatures. Some bacteria for example are able to live and grow in water springs at 80°C (Stetter *et al.*, 1990). In this thesis it was assumed that the SDFT is maintained at body core temperature and therefore equine tendon fibroblasts were grown in culture at a temperature of 37°C. This however may not be the case; on a frosty morning the subcutaneous temperature of the distal part of the equine limb may be as low as 11°C (Wilson, unpublished data). Thus, equine SDFT fibroblasts are subjected to a wide range of temperatures *in vivo* and this has implications for the repair process. Low temperatures may limit the healing process following partial rupture (Silver, 1973). The range of temperatures over which tendon fibroblasts are able to grow and divide and produce collagen could be studied *in vitro*. The advantage of hyperthermic resistance for SDFT fibroblasts is clear; the mechanism responsible for this resistance is however unknown. Further study on this particular tenocyte characteristic could be done using differential scanning calorimetry. In this system cells are heated and the temperature at which a particular endothermic peak occurs and the amount of energy involved is recorded. These peaks represent denaturation of a particular sub-cellular component. Cells would then be put back into culture and cell survival rates measured. The different endothermic peaks can be identified to a particular cellular component by carrying out a subcellular fractionation and repeating the calorimetry measurements. In this way it is possible to identify damage to a particular sub-cellular component at a particular temperature and separate sub-lethal from lethal cell damage.

When the nature of the resistance to hyperthermia is determined this could be exploited in training techniques. If for example heat shock proteins are involved a short gallop prior to the race may protect tendon cells from otherwise damaging levels of hyperthermia.

8.5 Control of Matrix Synthesis

An understanding of control of the amount and type of collagen production by tenocytes is crucial to the understanding of both adaptation and degenerative change within equine SDFT. Control may be exerted at one or more of several points along the series of events leading to appearance of collagen in the extracellular compartment. Molecular biological techniques provide a useful tool for the study of this control. The degree of transcription can be determined by measuring messenger ribonucleic acid (mRNA) levels within the cell. Translation of mRNA into polypeptide chains and assembly into collagen molecules can be determined by incubating with radiolabelled proline and measuring labelled intracellular collagen concentrations. The breakdown of collagen within the cell can be assessed by measuring the disappearance of a radiolabelled pool of collagen. Finally exocytosis of collagen can be determined by measuring radiolabelled collagen in the medium.

Control of collagen type is an interesting direction for the development of new drugs, which could be used in the treatment of partial tendon ruptures. "Adequan", a polysulphated glycosaminoglycan is currently under clinical trial for treatment of acute tendon injuries, has been found to stimulate the production of both collagen and glycosaminoglycans (Glade, 1990) although whether this affects collagen type is not known. Metrenperone, an S_2-blocker, has shown promising results in the treatment of clinical equine tendon injuries particularly in chronic cases where a large percentage of type III collagen persists (Williams, 1984). The mode of action of Metrenperone is not known but it is a peripheral vasodilator and therefore likely to increase blood flow to the tendon tissue. It may be that the resulting change in cellular biochemical environment is sufficient to alter collagen production towards type I. Any drug which could be shown to increase type I collagen synthesis while suppressing type III would have exciting implications for the treatment of tendon injuries.

An understanding of control mechanisms for proteoglycan synthesis could also be exploited in the treatment of tendon injuries. Glycosaminoglycans differ with fibril diameters and are likely to play a rôle in control of fibrillogenesis. At different stages of the repair process collagen aggregation could be controlled by stimulating production of a proteoglycan with a particular glycosaminoglycan sidechain. Alternatively, glycosaminoglycans could be introduced into the tendon by injection.

8.6 Effects of Training

Training might be expected to result in an adaptation resulting in a stronger tendon as for example occurs in muscle and bone. In chapter 2, however, it was shown that there were no differences in the matrix composition of equine forelimb flexor tendons, on the basis of the parameters measured, between horses with an active history compared with those that had led a more sedate life style. There are several possible explanations for this apparent lack of change in tendon material properties in response to a mechanical stimulus.

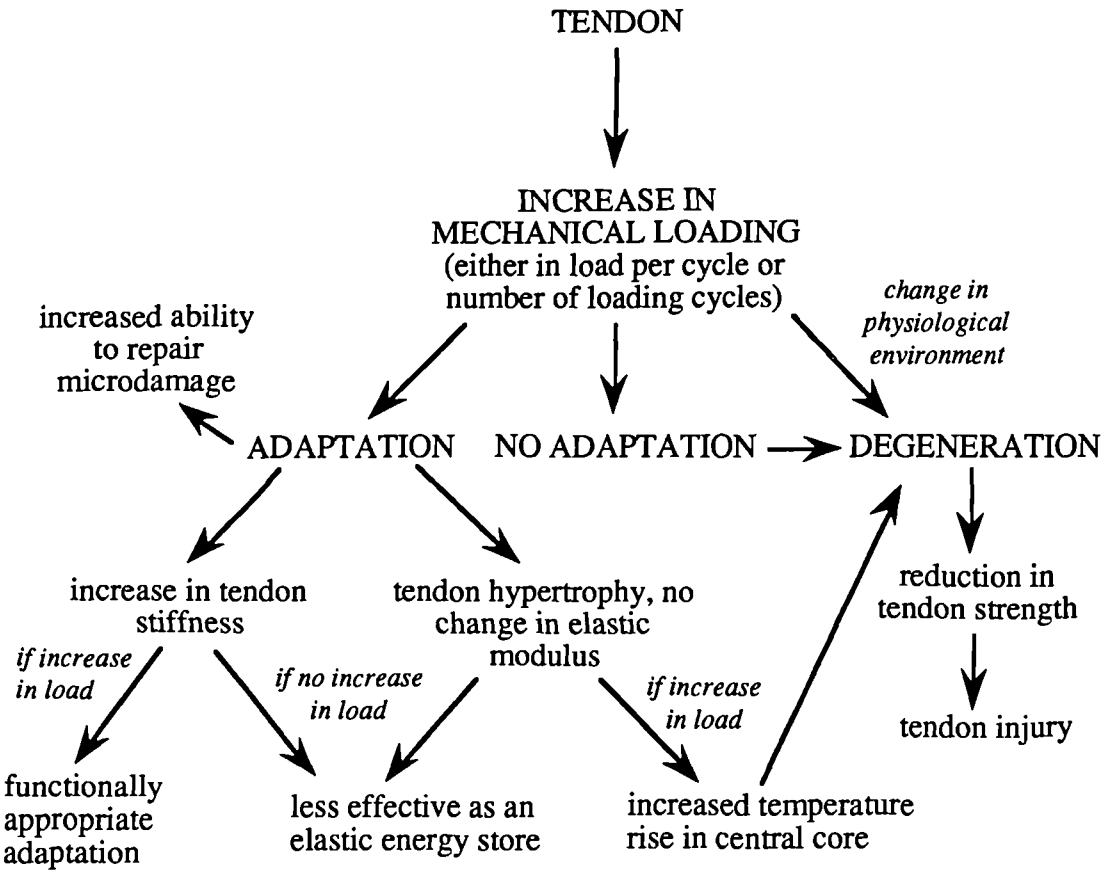


Fig. 8.2 Possible effects of mechanical loading on tendon properties.

First, tendons may be unable to adapt by increasing deposition of collagen and other non-collagenous matrix macromolecules due to a nutrient depravation. It was shown in chapter 4, contrary to previously published work, that tenocytes possess mitochondrial enzymes and carry out oxidative metabolism and therefore have an oxygen demand. It is likely that the poor blood supply and dense nature of the

extracellular matrix results in low oxygen tension and nutrient concentration in the immediate vicinity of the cells (Fackelman, 1973). Both hypertrophy and increased collagen turnover would demand increased protein synthesis. Therefore, the cellular biochemical environment may be a limiting factor in this process. It seems unlikely however that evolutionary change would result in a structure which is unable to adapt to its mechanical environment and thereby susceptible to injury.

The second possibility is that maximum adaptation within the SDFT tendon is attained with only a low number of loading cycles and further training therefore has no effect. This would imply that the SDFT has adequate material properties to carry out its mechanical rôle and a further increase in strength is not necessary. At first glance this seems unlikely considering the high incidence of failure in this tendon. The validity of this concept however, may be further explored by considering the function of the equine SDFT. As described in chapter 1, not all tendons have the same physiological function. In those tendons which serve to convert the contraction of a muscle into the movement of a joint, training would be expected to result in an increased mechanical strength of the tendon. This is because both bone and muscle increase in strength following training and tendon, which forms the link between the two, would be subjected to higher loads due to increased muscle power. The equine SDFT however, also acts as an elastic energy store and its main function is to release the stored energy lifting the fetlock and propelling it forward ready for the next stride thus, increasing the efficiency of high speed locomotion (Alexander, 1988). The tendon is loaded as the horse lands on the leg and the metacarpo-phalangeal joint extends, mainly due to the weight of the horse. The question which must be asked is: In a "racing fit" horse is this tendon subjected to higher loads? Considering that propulsive power in the "equine athlete" is provided by the hindlimbs, with the forelimbs contributing little, but acting as props to support the fore-end of the horse, there is no reason to believe that tendon load would increase as a result of training as the SDF muscle would not undergo hypertrophy. The forelimbs of the horse, however, serve to accelerate and decelerate the torso vertically and, due to the shorter stance phase at higher speeds, peak tendon load would increase. Fit horses, however, do not necessarily gallop faster but are able to maintain speed for longer. Therefore, there is no reason for the SDFT to respond by an increase in mechanical strength. However, horses engaged in intense exercise programmes, as when in race training, will be subjecting their SDFTs to an increased number of loading cycles. In bone, repetitive loading cycles can result in fatigue fractures (Nunamaker, 1987). Similarly in tendon an increase in the number of loading cycles may also result in an accumulation of microdamage to the matrix. Adaptations to training therefore may be expected to occur whereby the capacity of the tendon to repair such microdamage is increased. On reflection, the absence of a change in tendon properties following training, which might be expected to result in an increase in

ultimate strength, should not be interpreted as no response to training. As mentioned above, the hindlimbs of a horse are the powerhouse. Therefore, to test the theory that tendons adapt to perform their particular function better, could be tested by comparing the flexor tendons from the equine Thoroughbred forelimb with those from the hindlimb, in a group of trained versus control horses.

Third, in showjumping horses, where tendon loads may be high but repetitive cycles relatively low, SDFT injury is rare. In contrast, racehorses, particularly those performing over longer distances, suffer a high incidence of partial rupture to the SDFT. This provides further evidence that the observed degenerative change in the central core of the SDFT may be due to an inability to repair microdamage caused by repetitive loading cycles, or alternatively an inappropriate repair response. The results of chapter 3 suggest that the latter is the case. A reduction in collagen-linked fluorescence in the central zone of degenerated tendons suggests that collagen turnover is increased. Type I collagen however, is replaced with type III collagen resulting in a weaker matrix, in a similar manner to that following gross damage to the tendon (Williams *et al.*, 1984).

Finally, differences between the flexor tendons of exercised and non-exercised groups of horses may not have been apparent in this study due to an inaccurate assignment of horses to each of the groups. A history and description of the type of work undertaken by each horse was taken from the owner and this area is a possible source of error. Personal opinions differ on what may be described as light work only and what constitutes a vigorous training programme. A horse which some people may describe as a hack or riding horses may have been worked hard, and often with less consideration given to their degree of fitness and the surface on which they are exercised, compared to horses in a more serious training programme, therefore increasing the amount of wear and tear on their limbs. Also, due to conformational and physiological factors two horses carrying out the same exercise regime may put different strains on their musculoskeletal system.

To address this question of flexor tendon response to training one needs to start with a group of age-matched young horses straight from the breeder. These horses should not have been broken in and ridden, or tested for racing ability in anyway, and therefore would represent a true untrained group of horses. Half of these horses would then carry out a well defined programme of exercise similar to that which they would be subjected to if in a racing yard. The control horses would have only walking exercise. This type of experiment is currently underway within our department and should provide valuable material to answer some of the questions raised above.

If the above study reinforces the findings of this thesis i.e. that equine SDFT does not adapt to training, this should have a profound effect on the training of horses. It is common practice to give horses 6 weeks of walking and trotting exercise on a hard

surface. This is known as road work and is said, with no supporting evidence, to "harden the tendons". This may not only be of no value to tendon material properties but also be detrimental to joints. Training programmes should instead be designed to cause bone and muscle to adapt whilst causing minimum damage to tendons.

Advances in the prevention, treatment and rehabilitation of tendon injuries depends upon furthering the understanding of normal tendon composition and metabolism and the changes which occur with age. An understanding of the way in which tendon cells respond to biomechanical stimuli and their physiological environment will lead to a better understanding of the processes leading to tendon degeneration and a scientific rational for the treatment of tendon injuries once they occur.

8.7 Conclusions

1. Degenerative changes within the central core of the equine SDFT are characterised by an increase in type III collagen content and glycosaminoglycan content relative to the peripheral tissue and a high tissue cellularity. It was not possible in this study to determine whether these changes could be attributed purely to ageing or whether they were exercised related.

2. Mature tenocytes have mitochondrial enzyme activities and are capable of oxidative energy metabolism. Tenocytes therefore have an oxygen demand and a poor blood supply is likely to compromise cell function.

3. Compromising the production of ATP by aerobic pathways was not detrimental to tendon fibroblast cell function *in vitro*. However *in vivo*, where anaerobic capacity is not as great, hypoxia may limit cell activity particular in combination with hyperthermia.

4. Tendon fibroblasts display an increased resistance to hyperthermia relative to other fibroblast cell lines. Heat shock stimulates cell proliferation rates and collagen secretion into the medium.

8.8 Further studies

1. Analysis of extracellular matrix components and cellularity of age matched, trained versus control Thoroughbred horses.

2. Culture of explants of tissue from the above groups of horses to determine the amount and type of collagen and proteoglycan synthesis and differences between groups and central and peripheral tissue. In situ hybridisation on frozen sections of the same tendons to demonstrate any difference between central and peripheral tissue.

3. Scanning calorimetric techniques to determine the nature of thermal resistance. Immunohistochemical localisation of heat shock proteins in frozen sections from above studies.

4. Development of culture techniques with removal of growth factors to limit cell proliferation and lactate dehydrogenase induction. Further study of true effect of hypoxia versus compromise of energy metabolism by aerobic means using these cells.

5. Further studies, using the above cells, on the effect of biochemical environment on production of type I versus type III collagen and point at which control is exerted.

APPENDIX 1

Abbreviations

acetyl CoA - acetyl coenzyme A

ADP - adenosine diphosphate

AGE - advanced glycosylation end-product

AMP - adenosine monophosphate

ANOVA - analysis of variance

ATP - adenosine triphosphate

CNBr - cyanogen bromide

CS - chondroitin sulphate

CtS - citrate synthetase

CytO - cytochrome oxidase

DDFT - deep digital flexor tendon

DHLNL - dihydroxylysinonorleucine

DMEM - Dulbecco's modified Eagle's medium

DNA - deoxyribonucleic acid

DS - dermatan sulphate

EDTA - ethylenediamine tetra-acetic acid

$FADH_2$ / FAD - flavin adenine dinucleotide (reduced and non-reduced form)

FCCP - carbonyl cyanide p-trifluoromethoxyphenylhydrazone

FCS - foetal calf serum

GAG - glycosaminoglycan

GDH - glutamate dehydrogenase

HEPES - N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid

HHL - histidinohydroxylysinonorleucine

HLNL - hydroxylysinonorleucine

HP - hydroxylysylpyridinoline

HPLC - high-performance liquid chromatograpy

ICDH-NAD$^+$ - NAD$^+$ linked isocitrate dehydrogenase

KS - keratan sulphate

LDH - lactate dehydrogenase

LP - lysylpyridinoline

mRNA - messenger ribonucleic acid

MDH - malate dehydrogenase

MOPS - 3-(N-morpholino) propane-sulphonic acid

NADH / NAD$^+$ - nicotinamide adenine dinucleotide (reduced and non-reduced form)

OGDH - 2-oxoglutarate dehydrogenase

PBS - phoshate buffered saline

SDFT - superficial digital flexor tendon

SDH - succinate dehydrogenase

SDS-PAGE - sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SOD - superoxide dismutase

TBS - Tris buffered saline

TRIS - Tris(hydroxymethyl)aminomethane·HCl

VEGF - vascular endothelial growth factor

XO - xanthine oxidase

APPENDIX 2

Appendix 2 gives the details of horses used in each of the studies. All horses were half- to full-Throughbred and not smaller than 15 hh.

1. Horses tendons used in the comparison of extracellular matrix components of young (group 1), old un-exercised (group 2) and old exercised horses (group 3).

Horse	Material	Group	Age	History
1	SDFT & DDFT central & peripheral tissue	1	3	Lightly ridden only
2	SDFT & DDFT central & peripheral tissue	1	6	Hunted. Navicular disease and ringbone
3	SDFT & DDFT central & peripheral tissue	1	5	Lightly ridden only. Degenerative joint disease of fetlock
4	SDFT & DDFT central & peripheral tissue	1	3	Not ridden. Deformed face
5	SDFT & DDFT central & peripheral tissue	1	3	Not ridden. Fx. pelvis as a foal
6	SDFT & DDFT central & peripheral tissue	2	16	Not raced. Some eventing
7	SDFT & DDFT central & peripheral tissue	2	16	Hunted
8	SDFT & DDFT central & peripheral tissue	2	16	Show horse and broodmare
9	SDFT & DDFT central & peripheral tissue	2	12	Hunted
10	SDFT & DDFT central & peripheral tissue	2	13	Dressage horse
11	SDFT & DDFT central & peripheral tissue	2	16	Broodmare

Horse	Material	Group	Age	History
12	SDFT & DDFT central & peripheral tissue	3	12	Raced and hunted
13	SDFT & DDFT central & peripheral tissue	3	16	Raced, hunted, evented and team chased.
14	SDFT & DDFT central & peripheral tissue	3	16	Team chased and evented
15	SDFT & DDFT central & peripheral tissue	3	16	Team chased and evented
16	SDFT & DDFT central & peripheral tissue	3	15	Raced
17	SDFT & DDFT central & peripheral tissue	3	8	Raced

2. Horse tendons used for quantification of the different glycosaminoglycans in SDFT and DDFT (chapter 2).

Horse	Material	Age	History
1	SDFT & DDFT whole tendon	9	General purpose riding horse
2	SDFT & DDFT whole tendon	15	Hunter
3	SDFT & DDFT whole tendon	10	General purpose riding horse
4	SDFT & DDFT whole tendon	18	Broodmare
5	SDFT & DDFT whole tendon	20	Broodmare
6	SDFT & DDFT whole tendon	20	Racehorse

3. Horse tendons used for the comparison of extracellular matrix components in "normal" (N) and degenerated (D) SDFTs (chapter 3).

Horse	Material	Group	Age	History
1	SDFT central & peripheral	N	8	Showjumper
2	SDFT central & peripheral	N	9	Hunter
3	SDFT central & peripheral	N	14	Hunter
4	SDFT central & peripheral	N	8	Riding horse
5	SDFT central & peripheral	N	11	Hunter and general purpose riding horse
6	SDFT central & peripheral	N	4	Potential eventer
7	SDFT central & peripheral	D	4	Racehorse
8	SDFT central & peripheral	D	16	Racehorse
9	SDFT central & peripheral	D	14	Racehorse
10	SDFT central & peripheral	D	15	General purpose riding horse
11	SDFT central & peripheral	D	15	Hunter
12	SDFT central & peripheral	D	12	Racehorse

4. Horse tendons used for cell isolation and assay of key metabolic enzyme activities (chapter 4).

Horse	Material	Group	Age
1	SDFT central & peripheral	Y	2
2	SDFT central & peripheral	Y	1
3	SDFT central & peripheral	Y	3
4	SDFT central & peripheral	O	8
5	SDFT central & peripheral	O	14
6	SDFT central & peripheral	O	9
7	SDFT central & peripheral	D	3
8	SDFT central & peripheral	D	9
9	SDFT central & peripheral	D	15

5. Horse tendons used for cell isolation and measurement of glucose metabolism (chapter 4).

Horse	Material	Age
1	SDFT whole tendon	8
2	SDFT whole tendon	18
3	SDFT whole tendon	16

6. Horse tendons used for the culture of equine tendon fibroblasts (chapter 5).

Horse	Material	Age
1	SDFT central & peripheral	8
2	SDFT central & peripheral	9
3	SDFT central & peripheral	15
4	SDFT central & peripheral	6
5	SDFT - central & skin sample	15
6	SDFT - central & skin sample	20
7	SDFT - central & skin sample	15
8	SDFT - central	6
9	SDFT - central	12
10	SDFT - central	6

APPENDIX 3

Materials

All chemicals and biochemicals were obtained from BDH. In addition, L-cysteine·HCl, papain, Hoechst no. 33258, deoxyribonucleic acid (DNA) (calf thymus, sodium salt, highly polymerized), chondroitin sulphate A (bovine trachea), chondroitinase ABC, chondroitinase AC, chondroitin sulphate B (dermatan sulphate, porcine skin), trans-4-hydroxy -L-proline, cyanogen bromide (CNBr), bromophenol blue (sodium salt), TEMED (N,N,N',N'-tetramethylethylenediamine), rabbit anti-goat IgG FITC antibody, potassium borohydride, N,N-dimethylformamide, collagenase (type II), trypan blue solution, DL-dithiothreitol (DTT), ethylene glycol-bis-(ß-aminoethyl ether)-N,N,N',N',-tetraacetic acid (EGTA), pyruvic acid (sodium salt), 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), 2-oxoglutarate (disodium salt), phenylethylamine, antibiotics (streptomycin, 100 µg/ml; benzylpenicillin, 200 U/ml; neomycin, 50 U/ml), DL-isocitric acid (trisodium salt), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), arsenic acid (sodium salt), luciferase (firefly extract), hydrogen peroxide (30% solution), catalase (bovine liver), superoxide dismutase (human erythrocyte), β aminopropionitrile (monofumarate salt), L-ascorbic acid (sodium salt), phenylmethylsulfonyl fluoride, N-ethylmaleimide, pepsin (porcine stomach mucosa), polyoxyethylenesorbitan monolaurate (TWEEN 20), rabbit anti-goat IgG antibody (alkaline phosphate labelled), 5-bromo-4-chloro-3-indolyl phosphate (p-toluidine salt) and nitro blue tetrazolium were obtained from Sigma Chemical Company Ltd, Poole, Dorset, U.K. Glutaraldehyde (25%), paraformaldehyde, sodium cacodylate (sodium dimethyl arsenate), osmium tetroxide (2%), Taab 812 resin, Reynold's lead citrate and uranyl acetate were obtained from Taab Laboratories Equipment Ltd, Reading, Berkshire, U.K. Reagents for the LKB 4400 amino acid analyser were obtained from Pharmacia LKB Biochrom Ltd, Cambridge, England, U.K. Phosphate buffered saline tablets (Dulbecco A) were obtained from Unipath Ltd, Basingstoke, Hampshire, U.K. 1,9-dimethyl-methylene blue, Coomassie brillant blue G and D,L-glyceraldehyde were obtained from Aldrich Chemical Company Ltd, Gillingham, Dorset, U.K. Tissue-Tek and citifluor were obtained from Agar Scientific, Stansted, Essex, U.K. Goat anti-type I collagen antibody and goat anti-type III collagen antibody were obtained from Southern Biotechnology Associates, Inc., Birmingham, Alabama, U.S.A. CF1 cellulose was obtained from Whatman Biosysyems Ltd, Maidstone, Kent, U.K. Heptafluorobutyric acid and acetonitrile (HPLC grade) were obtained from Rathburn Chemicals Ltd, Walkerburn, Peeblesshire, Scotland, U.K. Bovine serum albumin (BSA) was obtained from Park Scientific Ltd, Northampton, U.K. Adenosine-5'-diphosphate (ADP) (disodium salt), ß-nicotinamide-

adenine dinucleotide (NADH) (disodium salt), oxaloacetate, acetylCoA, ethylphenolpoly(ethyleneglycolether)$_n$ (NP-40), ß-nicotinamide-adenine dinucleotide (NAD^+) (free acid, grade II), thymidine-5'-triphosphate (TTP) (tetrasodium salt), coenzyme A (CoA), adenosine-5'-triphosphate (ATP) (disodium salt) were obtained from Boehringer Mannheim UK, Lewes, East Sussex, U.K. Rotenone was a gift from Dr Andrew Halestrap, Department of Biochemistry, University of Bristol, U.K. D-[6-^{14}C] glucose (specific activity 52·8 Ci/mol), D-[5-^{3}H] glucose (specific activity 10·5 Ci/mmol), [methyl-^{3}H] thymidine (specific activity 25·0 ci/mmol), L-[U-^{14}C] proline (specific activity 0·26 ci/mmol) and Amplify were obtained from Amersham International plc, Aylesbury, Buckinghamshire, U.K. Fungizone (amphotericin B) was obtained from E.R. Squibb & Sons, Princeton, New Jersey. Hank's balanced salts (HBSS), Dulbecco's modified Eagle medium (DMEM), foetal calf serum (FCS) and trypsin solution (2·5%) were obtained from GIBCO, Life Technologies Ltd, Paisley, Scotland, U.K. Ultimagold scintillant was obtained from Canberra Packard Ltd, Pangbourne, Berkshire, U.K. Immobilon P membrane was obtained from Millipore (U.K.) Ltd, Watford, Hertfordshire. Equine collagen standards were a gift from Dr A.R.S. Barr, Department of Veterinary Surgery, University of Bristol, U.K. NRK 49F cells and tissue culture 60 mm plastic petri dishes were purchased from Flow Laboratories Ltd, Rickmansworth, Hertfordshire, U.K. 0·2 µm filters were obtained from Gelman Sciences, Northampton, U.K. Screw-top hydrolysis tubes were obtained from Corning Laboratory Service, New York, U.S.A. Sterilin tubes were obtained from Bibby Sterilin Ltd, Stone, Staffordshire, U.K. Falcon plastic, 24 well, tissue culture growth plates and Falcon tissue culture flasks were obtained from Becton Dickenson Labware, Lincoln Park, New Jersey, U.S.A. Kodak diagnostic film (XRP-6) was obtained from Phase Separations Ltd, Deeside, Clwyd, Wales, U.K.

APPENDIX 4

Publications in which parts of this thesis have appeared

Birch, H.L., Wilson, A.M. & A.E. Goodship (1992) Effect of exercise induced hyperthermia on tendon cells. Journal of Biomechanics (in press)

Birch, H.L., Bailey, J.V., Wilson, A.M. & A.E. Goodship (1992) Fibril diameters and glycosaminoglycan content differ in two equine flexor tendons with different functional rôles. Journal of Bone and Joint Surgery (in press)

Bailey, J.V.B., Birch, H.L., Bailey, A.J. & A.E. Goodship (1992) A morphological and biochemical comparison of the deep and superficial digital flexor tendon in the horse. Journal of Veterinary Surgery (in press)

Birch, H.L., Wilson, A.M. & A.E. Goodship (1993) Hyperthermia - A possible aetiological rôle in tendon degeneration. Transactions of the 39th Orthopaedic Research Society meeting, San Francisco.

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